The influence of mid-continent agricultural land use on the health and survival of commercially managed honey bee (*Apis mellifera* L.) colonies

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Dedication

To my grandmother Nora Smart Abernathy, and uncle Dan Buettner, who were so very interested in this project but passed before its completion.

Abstract

Commercial honey bee colonies were assessed in six apiaries that varied in their land use composition, in the Prairie Pothole Region of North Dakota over three years, 2010-2013. All colonies were transported to California to pollinate almonds each fall and were transported back to North Dakota each spring. The goal of the study was to determine the factors that most influenced, and thus predicted, annual survival of colonies in the different apiaries from summer through the following spring. The factors were grouped into three levels of analysis: 1) land use surrounding the North Dakota summer apiaries, including floral availability and pesticide exposure; 2) colony-level measures of population size, pollen and honey stores, queen status, and presence and prevalence of parasites and diseases; and 3) individual bee-level measures of nutritional physiology and immunity in 7-day old nurse bees collected from healthy colonies within each apiary. Results indicated that the area of uncultivated land (including CRP lands, pasture, grassland, flowering trees and shrubs, fallow land, hayland, and ditches) exerted a significant positive influence on the annual proportion of colonies surviving among apiaries. At the colony level, the amount of brood (pupae) in September and the mean pollen (g) collected per day over the summer correlated with higher annual survival. Higher *Varroa destructor* mite infestation levels in September were associated with reduced overwinter survival. Individual bee measures positively influencing survival included the expression level of vitellogenin in September and abdominal lipid stores in August. The expression level of lysozyme-2 in September was related to decreased apiary survival. A final, integrated model, incorporating all of the significant factors across the three levels, revealed that all, except *Varroa* levels, remained significant as

predictors of annual colony survival within apiaries. *Varroa* was actively and effectively controlled by the collaborating beekeeper; thus in this study was not an overall contributor to colony mortality. This is the first study to quantify the impact and importance of pollen nutrition; i.e., "pollen flow" from the level of landscape to the individual-bee, to the health and survivorship of colonies. The most significant predictors of health and survivorship across all three levels of analysis were all related to nutrition - beginning with abundant flowers located overwhelmingly in uncultivated lands. More and/or better forage led to greater honey production and pollen collection which in turn led to greater nutritional stores in individual bees, and an overall decreased immune response. The presence of quality and abundant forage surrounding summering locations support healthy, robust, and most importantly, surviving, colonies of honey bees.

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CHAPTER 1 – INTRODUCTION

Factors influencing the health and survival of honey bee (*Apis mellifera* L.) colonies

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1.1 Overview of colony losses and land use in upper Midwest region of the U.S.

The managed European honey bee (*Apis mellifera*) is of critical importance towards insuring the present and future availability and security of food in the United States and worldwide. In the U.S. alone, this single species of bee is responsible for the pollination of around 130 crops (McGregor, 1976), adding an estimated $15 billion annually to the value of honey bee-pollinated crops (Morse and Calderone, 2000). While recent declines in pollinator species have been documented across the globe, the phenomenon of sustained, severe losses of managed, commercial honey bee colonies (and the separate but overlapping phenomenon of Colony Collapse Disorder, CCD) has mainly been confined to North America and parts of Europe (NRC, 2006; vanEngelsdorp et al., 2008; Potts et al., 2010). Annual losses for commercial beekeepers in the U.S. have hovered around 30% since 2006-2007, with a low of 22.3% in 2011-12 and a high of 40.1% in 2012-13 (vanEnglesdorp et al., 2007; vanEnglesdorp et al., 2008; vanEnglesdorp et al., 2010; vanEnglesdorp et al., 2011; vanEnglesdorp et al., 2012; Spleen et al., 2013; Steinhauer et al., 2014).

Several factors have been implicated as potentially contributing to stress and loss of honey bee colonies (e.g. Cox-Foster et al., 2007; vanEngelsdorp et al. 2009a; vanEngelsdorp et al., 2013) that may be broadly categorized into three groups acting alone or in conjunction with each other: 1) Poor diet brought on by inadequate and/or

deficient forage resources (Naug, 2009b; Alaux et al., 2010a; DeGrandi-Hoffman et al., 2010; Alaux et al. 2011; Di Pasquale et al. 2013), 2) exposure to environmental and in-hive pesticides (Johnson et al., 2010; Mullin et al., 2010; Boncristiani et al., 2012; Pettis et al., 2012; Wu et al., 2012; Johnson et al., 2013a; Johnson et al., 2013b), and 3) pests, pathogens, and microbes (Amdam et al., 2004a; Cox-Foster et al., 2007; Higes et al., 2008; Antunez et al., 2009; Mayack and Naug, 2009; Alaux et al., 2010b; Zhang et al., 2010; Chaimanee et al., 2012; Martinson et al., 2012). No single factor has been shown to occur in all cases of failing colonies, and therefore, a more complicated and nuanced picture of the dynamics occurring inside the hive has emerged – one in which many factors interact at various times of the year and lead to colony failure (Runckel et al., 2011; Pettis et al., 2012; Wu et al., 2012; DeGrandi-Hoffman et al., 2013; vanEngelsdorp et al., 2013).

Portions of the upper-Midwestern states of North Dakota, South Dakota, Montana, and Minnesota have historically acted as an unofficial "bee refuge" for a large proportion of the managed, commercial honey bee colonies throughout the growing season. This region hosts around 1 million commercial honey bee colonies from May-October every year, representing approximately 40% of the total U.S. managed, commercial pool of honey bee colonies (USDA, 2014b). Colonies brought to this region of the country for the summer have done very well historically (in terms of honey production) due, in large part, to an abundance of nectar and pollen-producing flowers present throughout the growing season. Critical regional land use categories supporting honey bee colonies include livestock-grazed pastures, oilseed, hay, and nitrogen-fixing crops, and more recently, conservation reserve program (CRP) lands, supporting blooms

of perennial clovers and alfalfa (blooming Mid-July through September), canola (blooming early June), sunflower (blooming late July through August), perennial shrubs (Gallant et al., 2014), and "weeds" such as sweet clover (*Melilotus* spp.).

Steep declines in acreage of the above types of land use (alfalfa, canola, sunflower, CRP) have occurred across the Great Plains region over the last decade; while concurrent acreage planted in non bee-utilized crops such as corn and soybeans has sharply increased (USDA, 2000; USDA, 2014a; Gallant et al., 2014). The expansion and increasing intensity of corn and soybeans brought on by relatively high commodity prices (Table 1.1) are particularly alarming given the large proportion of beekeepers aggregating in the region each summer. This, coupled with the fact that abundant bee forage in this region is crucial for both honey production and the success of summering colonies going into the winter with future intentions of producing queens, package bees, and providing pollination services, highlights the importance of clearly delineating outcomes derived from varying agricultural land use during the summer on honey bees.

"North Dakota and California are getting closer all the time"

- J. Miller, ND commercial beekeeper

Land use, and the resulting resource availability for honey bees in the Upper Midwest, are emerging as critical factors in efforts to secure and conserve the available and necessary populations of honey bees in the region that go on to affect many other downstream parts of the beekeeping industry, including pollination of almonds in California, pollination of other crops across the U.S., and package bee and queen

production in the southern states. Commercial honey bee colonies that are located in the upper Midwest during the summer are typically moved south (e.g. Texas and Louisiana for queen and package bee production and/or California for almond pollination) or indoors (e.g. climate-controlled potato sheds in Idaho) during the winter. The present study examines the health and survival of commercial honey bee colonies used for early spring California almond pollination - the plant with which present day U.S. commercial beekeepers have the closest economic relationship.

Commercial cultivars of almond are self-incompatible, requiring the presence of rows of pollenizers within orchards. Flowering occurs from February-March each spring, and since daytime temperatures above approximately 57˚ F are required for pollinating insect flight, the central California Sacramento and San Joaquin valleys are ideal locations for growing almonds. California is the only U.S. state producing almonds commercially and accounts for approximately 80% of the world's almond crop (Boriss and Brunke, 2005). The 2012 almond crop totaled approximately 2 billion pounds and was valued at $4.3 billion (USDA, 2013). As such, each year approximately 1.5 million of the 2.5 million available colonies nationwide undertake the long journey to the central valleys of California. The present-day 800,000 bearing acres of almonds are 100% dependent on the pollination that they receive from insects, with *Apis mellifera* providing the bulk of pollination services. As such, beekeepers have been compensated at a higher rate for pollinating almonds than any other crop, particularly in recent years, bringing in around $140-160 for 6 frames colonies and $170-200 for 8-10 frames colonies (Mader et al., 2010; Traynor, 2014).

Perhaps surprisingly, land use as an indicator of honey bee health and survival, and landscape-wide honey bee foraging patterns have only been considered thoroughly in few studies (e.g. Naug, 2009; Couvillon et al., 2014). These and other similar studies tend to focus on honey production, pollination services, and foraging distances of colonies positioned around various crops and land use features (Beekman and Ratnieks, 2000; Steffan-Dewenter and Kuhn, 2003; Ricketts, 2004; Odoux et al., 2012; vanEngelsdorp et al., 2013; Gallant et al., 2014), but fail to acquire a detailed account of the health and survival of colonies embedded in a typical migratory beekeeping operation in response to specific landscapes to which the colonies are exposed.

Therefore, a key question yet to be addressed heretofore in a large field study is the degree to which land use around apiaries directly leads to varying outcomes for commercial honey bee colonies in those landscapes. Here (in Chapters 2-5) I identify particular types of land use within the larger agricultural matrix that are associated with high and low apiary mortality. Further, I identify specific measures at the colony and individual bee levels that coincide with overall landscape quality and apiary survival based on land use patterns.

1.2 Honey bee nutrition

1.2.1 Protein

Honey bees rely on the availability of pollen for the amino acid, lipid, vitamin, and mineral components of their diet (Brodschneider and Crailsheim, 2010). The same 10 essential amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) required in the diets of vertebrates are

likewise needed by honey bees (de Groot 1953), which they acquire via direct fresh pollen-feeding, consumption of bee bread (pollen containing beneficial microbes mixed with honey and gland secretions), and/or via trophallaxis. Thus, while pollen contains other dietary components required by honey bees (lipids, vitamins, and minerals), pollen quality is generally defined as the crude protein content and/or relative composition of amino acids present in the pollen (Crailsheim 1990). Various plant sources of pollen may then be considered of higher or lower value to honey bees based on such levels, however, considerable variation exists among the results of such studies (Todd and Bretherick, 1946; Standifer, 1967; McCaughey et al., 1980; Evans et al., 1991; Shen, 1992; Pernal and Currie, 2000; Somerville, 2005a; Somerville, 2005b; Somerville and Nicol, 2006; Szczesna, 2006; Nicolson and Human, 2013).

Honey bees prefer pollen blends to single source pollens in certain cases (e.g. Schmidt, 1984; Schmidt et al., 1995) but not in others (e.g. Campana and Moeller, 1977; Alaux et al., 2010a). Further, various pollens and pollen mixes differentially affect survival in a lab setting (Schmidt et al, 1987; Schmidt et al., 1995; Di Pasquale et al., 2013). For example, Schmidt et al. (1987) fed 25 single source and 4 pollen blends to caged bees and observed survival over 60 days. Certain monofloral sources (e.g. *Populus* spp., *Rubus* sp.) and a five-pollen blend were associated with greatly improved longevity, greater consumption, and higher % protein content, while other single source pollens (e.g. *Ambrosia* sp., *Typha* sp., and *Kallstroema* sp.) decreased survival below even sucrose controls, were consumed less (except *Ambrosia* sp.), and had lower % protein content (Schmidt et al., 1987). In another study, sunflower (*Helianthus annuus* L.) and canola (*Brassica napus* L.) pollen were fed to caged bees and consumption and survival were

measured (Schmidt et al., 1995). Relative consumption and survival were the greatest for the canola pollen-fed bees. Sunflower pollen was also preferred by the bees (greatest cumulative consumption), but survival was not impacted relative to the pollen blend (Schmidt et al., 1995). Finally, Campana and Moeller (1977) determined that sweet clover (*Melilotus* spp.) was most preferred by honey bee colonies (highest rate and total consumption) compared to other single source and pollen blends, and further, *Melilotus* was found to contribute to the greatest area of brood production (Campana and Moeller, 1977).

Physiologically, results from laboratory studies in which varying pollen and/or percent crude protein diets were fed to worker honey bees indicate that, compared to sugar syrup controls, high protein diets result in greater nutritional stores and a decreased immune response, i.e. a quieter (non-activated) immune system (Alaux et al., 2010a; Alaux et al., 2011). Varying pollen and pollen substitutes also differentially affect bee responses to pathogen and parasite infection (DeGrandi-Hoffman et al., 2010; Di Pasquale et al., 2013). For example, longevity after parasite challenge (microsporidian, *Nosema ceranae*) was enhanced when worker bees were fed either a high quality single source or mix of pollens compared to lower quality pollen and sugar syrup controls (Di Pasquale et al., 2013).

Fresh and stored pollen and bee bread are consumed by young adult nurse bees to develop large glands in their heads that in turn produce and secrete a proteinaceous substance called “brood food”. The substance is actually a mixture of secretions from two glands in the head, the hypopharyngeal and mandibular glands (Winston, 1987; Gilliam, 1997; Vasquez and Olofsson, 2009). These secretions are successively fed to

developing immature bees by adult nurse bees and can easily be seen as milky white pools in the bottom of cells containing larvae. Bees of nursing age that are protein restricted and older bees beyond nursing age do not have well-developed hypopharyngeal glands (Crailsheim and Stolberg, 1989). As such, the size and protein content of these glands have bee used as diagnostic indicators of bee age, colony demography and colony growth (Huang et al., 1994; Huang and Robinson, 1996; Sagili and Pankiw, 2007; Wegener et al. 2009), and of overall health in response to varying nutrition, pathogen challenge, and pesticide exposure (Standifer, 1967; Crailsheim, 1990; Keller et al., 2005a; DeGrandi-Hoffman et al., 2010; Alaux et al., 2012; Coby-Harris et al., 2014).

Pollen consumption increases in adult workers until they reach nursing age, around 6-16 days post-eclosion (Winston 1987), at which time a decrease in pollen consumption is observed (Crailsheim et al. 1992). This decrease in pollen consumption is associated with myriad physiological and behavioral changes linked to the development of worker honey bees including: increasing juvenile hormone titers and a concurrent decline in levels of the storage protein vitellogenin, a general decline in immune competence, and the switch to "outside tasks" such as guarding the colony entrance, embarking on orientation flights, initiating foraging behavior, and ultimately, senescence (Huang and Robinson, 1996; Amdam and Omholt, 2002; Amdam et al., 2003; Amdam et al., 2004b; Amdam et al., 2005a; Nelson et al., 2007; Amdam et al., 2011).

The phospho-glycolipoprotein vitellogenin (Vg) plays multiple roles in honey bee nutrition, immunity, stress resistance, behavioral development, and longevity (Amdam and Omholt 2002; Seehuus et al., 2006; Marco Antonio et al., 2008; Munch et al., 2008; Nilsen et al., 2011). Vitellogenin, synthesized in trophocytes of the fat body and

circulated in the hemolymph, is a female-specific "yolk" storage protein that is taken up by developing oocytes of queen bees. In sterile honey bee workers, however, Vg is produced despite the lack of egg production, and is the most common protein found circulating in the hemolymph, though it is also found in the fat body and hypopharyngeal glands of workers (Fluri et al., 1982; Amdam et al., 2011).

Production of Vg in workers increases 2-3 days post-eclosion, and reaches a peak within the next 7-9 days at which time hemolymph titers stabilize (Fluri et al., 1982; Engles et al., 1990; Amdam and Omholt, 2002). Titers of Vg decrease rapidly in bees upon initiation of foraging behavior during the summer. Interactions between Vg, JH, and bee nutritional state, including signaling molecules such as insulin-like peptides (ILPs), are thought to participate in the behavioral maturation and longevity of the honey bee (Corona et al., 2007). Briefly, Corona et al. (2007) hypothesize that a high nutritional state leads to decreases in ILPs in the brain and a similar decrease in insulin receptors and downstream kinases leading to the nuclear translocation of forkhead box protein O (FOXO) that suppresses JH synthesis and, as a result, no suppression of Vg occurs. Importantly, other researchers have shown that abdominal expression of both Vg and ILP1 (as opposed to brain expression as in Corona et al., 2007) increase during amino acid supplementation (Nilsen et al., 2011).

High levels of vg are associated with young nursing bees and long-lived overwintering bees (Amdam et al., 2004b; Amdam et al., 2005a). Workers eclosing in late fall that are exposed to declining larval rearing and foraging activities are known as overwintering diutinous bees. These long-lived and stress-resilient individuals accumulate and maintain relatively high titers of Vg in the hemolymph and fat body

throughout the winter (Amdam and Omholt, 2002; Amdam et al., 2011). Vitellogenin has been previously utilized as a biomarker, indicative of an improved nutritional state and extended longevity (Alaux et al., 2011; Coby-Harris et al., 2014), and as a proxy relating pollen quality to bee nutritional physiology (Di Pasquale et al., 2013).

Protein and lipid production, including Vg, primarily occur in the insect fat body. This invertebrate organ has been analogized to the vertebrate liver and adipose tissue, exerting effects on both nutrition and immunity (Wheeler and Robinson, 1014). The organ exists as a single cell layer, lining the inner surface of the abdomen and is composed of trophocytes (sites of vg synthesis) and oenocytes (sites of lipid synthesis) (Roma et al., 2010; Amdam et al., 2011). The relative mass of the honey bee fat body has been previously used as an indirect proxy for age and nutritional state (Toth et al., 2005; Alaux et al., 2010; Ament et al. 2011), immunocompetence (Wilson-Rich et al., 2008) and longevity (Ellers, 1996; Duoms et al., 2002).

1.2.2 Carbohydrates

Nectar is the carbohydrate source of honey bees and, during the growing season, is primarily used to power the large flight muscles that dominate the thorax of adult foraging-aged honey bees. Nectar, stored in the returning forager honey crop (specialized portion of the foregut), is regurgitated to receiver bees at the hive that pack the nectar into cells. This process inoculates nectar with enzymes (invertase, diastase, glucose oxidase) that facilitate the breakdown of the sugars present in the nectar into monosaccharides. Additionally, before nectar is capped, the water content of the nectar is lowered to approximately 16-20% via wing fanning to prevent fermentation (Brodschneider and

Crailsheim, 2010). When the water content of the nectar is below 18.6% moisture it may be truly referred to as honey. Regurgitated nectar and honey are also added to pollen before it is packed into cells, facilitating the conversion of pollen to bee bread; a process assisted by beneficial microorganisms, protecting the stored pollen from pathogenic microbes and providing a more nutritionally-available source of protein (DeGrandi-Hoffman et al., 2009a; DeGrandi-Hoffman et al., 2009b; Vasquez and Olofsson, 2009; Anderson et al., 2014). Excess honey is stored for later consumption during times of dearth and for over-wintering. Honey bee colonies used by beekeepers for pollination services and honey production are routinely maintained in two deep brood chambers and honey boxes called "supers" are added above as needed. In the fall, supers are removed and the honey extracted from the combs by the beekeeper, while honey packed into cells of combs within the two deep boxes remains with the colony as a stored energy resource over the winter.

Honey bees rapidly respond to the availability of food resources in their environment (Seeley, 1995). This rapid up-regulation of foraging effort in response to the ephemeral availability of pollen and nectar is accomplished through the process of recruitment (Seeley, 1995). Further, honey bees can discern and specifically choose patches of more desirable flowers based primarily on the quantity and quality of their sugar rewards (Visscher and Seeley, 1982; Seeley, 1995). In addition to their ability to rapidly recruit additional foragers to resource sites, another characteristic that makes honey bees successful is their ability to rapidly build in population size within a given colony over time (Bodenheimer, 1937; Sakagami and Fukuda, 1968; Fukuda, 1983). This enables honey bees to carry out pollen and nectar collection (i.e. pollination) via

overwhelming numbers despite the greater efficiency of certain other pollinators on a per bee basis (Delaplane and Mayer, 2000; Stubbs and Drummond, 2001; Bosch and Kemp, 2002; Javorek et al., 2002), particularly during times of the year when other non-*Apis* bees are largely unavailable (e.g. February-March almond pollination).

1.3 Land use and wild pollinators

Studies of land use on wild and native bee diversity and abundance are much more predominant than those dedicated to honey bees, perhaps due to the relative stationary and localized distributions of wild bees. Such studies tend to be specific crop/plant pollination-focused (Stubbs and Drummond, 2001; Javorek et al, 2002; Ricketts, 2004; Shuler et al., 2005; Greenleaf and Kremen, 2006a; Winfree et al., 2008; Julier and Roulston, 2009; Holzschuh et al., 2011), highlight native and wild bee importance in enhancing fruit/seed set (Morandin and Winston, 2005; Klein et al., 2007) or emphasize their role in insuring against managed honey bee losses (Klein et al., 2007; Winfree et al., 2007; Winfree, 2008; Garibaldi et al., 2013). Overall, wild pollinators may be considered as bioindicators, relating the health or stress of an ecosystem, the suitability of a habitat given varying pollinator life histories, and the effects of habitat modification and pesticide exposure on pollinators (Kevan, 1999).

Long-term global trends in pollinator-dependent crop production indicate that no current pollinator shortages occur, but an increasing dependency on insect-pollinated crops could lead to a future lack of pollinators for certain crops and in certain regions (Aizen et al., 2008; Aizen and Harder, 2009; Gallai et al., 2009). Large-scale studies on agricultural practices indicate a negative effect of agricultural intensification, anthropogenic disturbance, and habitat fragmentation on wild bee diversity and

abundance (Morandin and Winston, 2005; Klein et al., 2007; Ricketts et al., 2008; Winfree et al., 2009; Le Feon et al., 2010). Certain land use types (e.g. pastureland, natural, and semi-natural habitats) located within larger agricultural mosaics may contribute to increases in wild bee presence and therefore pollination services for certain cultivated crops dependent on bee pollination (Greenleaf and Kremen, 2006b; Morandin et al., 2007; Jauker et al., 2009; Le Feon et al., 2010). However, demand for pollinators in certain cultivated crops like canola may contribute to a dilution of wild bees available for the pollination of concurrently blooming native plants (Holzschuh et al., 2011).

1.4 Non-native species

Apis mellifera, initially introduced to the New World by the English and Spanish in the 17th century, is a non-native species to the U.S. (Sheppard, 1989). As such, it is not particularly surprising that the European subspecies most widely kept in the U.S. (*A.m. ligustica*, *A.m. carnica*) might prefer introduced European and Asian species to native North American plant species. Regardless of preference, many of the cultivated plants grown on U.S. soil are, in fact, non-native as well (e.g. citrus, apples, melons, almonds, rapeseed, alfalfa, soybean). Interestingly, recent fossil evidence now indicates that honey bees were, in fact, endemic to North America dating back to the Middle Miocene (approximately 10-15 mya) before going extinct in the New World (Engel et al., 2009). The status of modern-day managed honey bees as non-native species in North America continues to be a subject of certain contention within communities concerned with the promotion and conservation of native ecosystems. The preference of honey bees for nectar and pollen from certain non-native species may enhance the ability of those introduced species to outcompete native plants in some settings (Morales and Traveset,

2009; Moron et al., 2009). Research focused on native plant species suitable for supporting both native pollinators and honey bees in terms of nutrition and honey production is active and on-going.

When considering the potential effects of non-native plant species on plant-pollinator networks, plants should be considered on a species by species basis as effects of certain non-native species may range from direct competitors with native plants for pollinators to facilitators of pollinator visits to native plants (Bjerknes et al., 2007; Bartomeus et al., 2008). Two critical species for honey bees in the Great Plains Region, white and yellow sweet clover (*Melilotus officinalis* and *M. albus,* native to Europe and Asia), may act as either facilitators or competitors depending on the habitat and plant community in which they are found (Van Riper and Larson, 2009). Still another study found *Melilotus* spp. to be attractive to many native bees, potentially increasing the carrying capacity of the environment and population sizes of native bees, including some specialists of closely related plants species (Tepedino et al., 2008). Further, *Melilotus* spp. will support high quality populations of the alfalfa leafcutter bee (*Megachile rotundata*), which has in turn been shown to be an effective pollinator of the two plant species (Richards, 2003).

On the other hand, a European study found non-native goldenrods (*Solidago canadensis* and *S. gigantea*) to strongly negatively affect wild bee diversity and abundance, and also negatively influence native plant diversity (Moron et al., 2009). However, non-native species may offer other benefits to conservation efforts, such as providing supplemental food sources to native species, substituting for endangered or extinct species, and providing other desirable functions in terms of ecosystem services,

such as soil nitrogen fixation (Van Riper and Larson, 2009; Schlaepfer et al., 2011). Additionally, non-native species may be particularly adept at persisting in environments susceptible to rapid changes, such as regions experiencing increasingly intensive agriculture or those expected to undergo climate change (Schlaepfer et al., 2011).

1.5 Pesticides

An average of 7 different pesticides are present in each pollen load of a returning forager honey bee (Mullin et al., 2010). The ready-and-willingness of individual forager honey bees from a given colony to visit many diverse plants brings them (and upon their return, bees in the nest) in contact with chemical contaminants present in the environment (in the forms of insecticides, fungicides, and herbicides applied to crops) (Mullin et al. 2010; Johnson et al. 2010; present study), as well as the internal hive environment (in the forms of current and residual acaricides for the control of the devastating parasitic mite, *Varroa destructor*) (vanEngelsdorp et al. 2009a; Johnson et al. 2009, Boncristiani et al., 2012; Johnson et al. 2013b).

Efforts to restrict the use of agricultural chemicals during blooms have addressed some of the exposure problem, however the residual activities of some pesticides have not been fully addressed (Johansen and Mayer, 1990). Newer technologies including the use of genetically modified crops and systemic neonicotinoid and phenylpyrazole insecticides have decreased grower reliance on older, highly toxic chemistries (to humans and pollinators), however the widespread use of these newer types of controls, systemic pesticides in particular, have raised new questions, particularly regarding their sub-lethal effects on pollinators (Yang et al., 2008; Aliouane et al., 2009; Wu et al. 2011; Pettis et al. 2012; Wu et al. 2012; Feltham et al., 2014).

1.5.1 In-hive miticides

Since the arrival of the *Varroa* mite, and to a lesser extent the tracheal mite (*Acarapis woodi*), to the U.S. in the 1980s, beekeepers have found it necessary to frequently treat their colonies with a variety of miticides. In 1987 the pyrethroid, *tau*-fluvalinate, was the first synthetic miticide registered for use in honey bee colonies under section 18 of FIFRA (Federal Insecticide, Fungicide, and Rodenticide Act) as an emergency use product (Ellis et al., 1988; Johnson et al., 2010).

As mites developed resistance to fluvalinate relatively quickly (Lodesani et al., 1995; Elzen et al., 1998), coumaphos, an organophosphate, was granted section 18 status in 1999 for use in honey bee colonies against *Varroa* mites and also small hive beetles (*Aethina tumida* Murray) (Johnson et al., 2010). While initially effective against fluvalinate-resistant mites, coumaphos-resistance was observed as early as 2001 (Elzen and Westervelt, 2002; Pettis, 2004). Further, coumaphos exposure has known negative effects on honey bee queens and drone sperm viability (Haarmann et al., 2002; Pettis et al., 2004; Burley et al., 2008). High residue levels of both fluvalinate and coumaphos, and their metabolites, continue to be commonly detected in wax comb and stored pollen despite a decades-long paucity in use of the compounds, and regular replacement of old combs by beekeepers (Mullin et al., 2010; Wu et al., 2011; present study).

In 1992 a formamidine pesticide, amitraz, was registered under section 18 for in-hive use against *Varroa* mites. However, the product was subsequently removed from U.S. markets for honey bees in 1994 after some reports of colony losses after treatment. Despite this removal, the product remained available in the U.S. as a veterinary miticide, but not for honey bee colonies. Amitraz metabolites (2,4 Dimethylphenyl formamide

DMPF) continue to be detected in beeswax, suggesting its continued use as a miticide (Johnson et al., 2010; Mullin et al., 2010; Wu et al., 2011). In 2013, amitraz was registered for conditional use, this time under section 3 of FIFRA, for use in honey bee colonies against *Varroa* mites, though resistance has already been exhibited in the U.S. (Sammataro et al., 2005). An additional chemical control, the pyrazole acaricide fenpyroximate, was introduced to the U.S. market in 2007, again, under section 18 registration for use against *Varroa* mites, however the original formulation is no longer available for use on honey bees.

As the efficacies of synthetic miticides have declined, and harmful effects incurred on bees have become clear, use of natural products have become more widespread, particularly for small-scale beekeepers. Fumigant products containing plant-derived essential oils such as thymol (oil of thyme) and menthol, registered under section 3 of FIFRA, are available to control *Varroa* and tracheal mites (Johnson et al., 2010). Such chemicals are recognized as generally safe for human consumption, though the same may not be true for honey bees, as some studies have shown thymol to effect the bees' removal of brood and increase queen mortality (Whittington et al., 2000; Floris et al., 2004; Johnson et al., 2010).

Beekeepers additionally have access to organic acid miticides such as formic and oxalic acids. Formic acid is registered in the U.S. under section 3 of FIFRA and available as a fumigant varroacide (Johnson et al., 2010). Formic acid, while being an effective varroacide, is known to decrease adult worker longevity and brood survival, and when applied to colonies in the spring, may cause queen loss or slow colony growth (Fries, 1991; Underwood and Currie, 2003; Johnson et al., 2010; Giovenazzo and

Dubreuil, 2011). Oxalic acid is registered for use in honey bee hives against *Varroa* mites in Canada and Europe, but not in the United States. It is, however, readily available on hardware shelves in the U.S. in the form of wood bleach; a fact likely contributing to the unwillingness of producers to undergo the costly and lengthy process of registration through the EPA for use as a varroacide in honey bee colonies in the U.S., although the EPA apparently has future intentions to register it as a *Varroa* control in 2015 (J. Pettis pers. comm.). Oxalic acid has been shown to be highly effective when trickled in sugar syrup over honey bees in cool climates during brood breaks (Aliano and Ellis, 2008; Canadian Honey Council, 2005). Prolonged exposure of colonies to oxalic acid may result in queen and sealed brood losses (Higes et al., 1999).

Overall, beekeepers continue to struggle to effectively suppress colony mite populations. There is a definite concern of the future efficacy of current pesticide chemistries and the lack of forthcoming new products in the pipeline as mites have historically quickly evolved resistance in the face of intense chemical pressure inside the hive. More research is needed on treatment regimes involving timing and rotation of chemistries to limit mite resistance, while also considering feasibility and untended consequences such as interactions and synergies that may occur inside various hive substrates and products.

1.5.2 Environmental pesticide exposure

Beekeepers and scientists alike are well aware of the dangers of pesticide exposure in controlled lab studies, however, measuring effects in a field colony setting has proven to be more difficult and somewhat contradictory, ranging from no detected

residues/no effect (Schmuck et al., 2001; Chauzat et al., 2009; Nguyen et al., 2009) to high residue levels and dramatic effects (Johnson et al., 2010; Krupke et al., 2012). The likely underlying cause of discrepancies among such studies is due to the fact that a given dose fed or applied to the surface of a bee in the lab is not necessarily analogous to the experience of a bee in the field or hive when they come in contact with pesticides. This difference is further exacerbated due the resource hoarding/storage behavior of bees such that exposure of foraging bees to contaminated food is spacio-temporally uncoupled from exposure of bees inside the hive, and further, pathways of exposure of bees in the hive depend on the point in development of a given bee when she is exposed to a chemicals. Effects of environmental pesticide exposure also depend on such factors as colony size, the co-occurrence of pests and pathogens, and the timing of exposure, including time of year and whether exposure is acute or chronic. Importantly, the lipophilic nature of beeswax makes it an excellent sink for many pesticides. A highly contaminated pesticide load profile can have myriad effects on colonies and individual bees alike, including everything from sublethal effects to death (Yang et al., 2008; vanEngelsdorp et al., 2009b; Mullin et al., 2010; Wu et al., 2011; Wu et al., 2012).

Sub-lethal effects of pesticides include those that negatively affect memory, learning, cognition, longevity, susceptibility to diseases, and colony development (Yang et al., 2008; Aliouane et al., 2009; Wu et al. 2011; Pettis et al. 2012; Wu et al. 2012; Feltham et al., 2014). The issue of pesticide exposure becomes exponentially more complicated when one considers that not only are there hundreds of active ingredients potentially contributing to bee deaths and sub-lethal effects outright, but also the interactions and synergies that may occur in the lipophilic internal environment of the

hive (Colin and Belzunces, 1992; Pilling and Jepson, 1993; Brobyn, 1999; Schmuck et al., 2003; Johnson et al. 2009; Gill et al., 2012), as well as the discovery that additives (i.e. adjuvants) not screened for honey bee toxicity may also be contributing to lethal and sub-lethal effects on bees (Johnson et al. 2013a).

Pesticide exposure in the external field environment and internal hive environment can have profound effects on both colonies and individual bees. Foraging honey bees may come into contact with both contaminated nectar, pollen, and water in the field. It is illegal for applicators to spray bee-pollinated crops during the day while blooms are present. However, pesticide label laws and recommendations are not always strictly adhered-to by landowners, land managers, farmers, or beekeepers (as it pertains to the control of *Varroa* mites and other parasites and diseases). Further, drift of agricultural pesticides may occur onto bee-visited flowers outside the margins of fields. Such bee-utilized "weeds" contain high levels of at least one systemic neonicotinoid insecticide, clothianidin, originating from dust during corn planting or via movement through the soil (Krupke et al., 2012). This same pesticide was detected at high levels in corn planter dust, in dead bees outside of colonies, in pollen loads of returning foragers, and in pollen stored inside bee colonies (Krupke et al., 2012).

Exposure to pesticides alone create many problems for bees, however, recently they have been discovered to interact with pests and pathogens of honey bees. For example, high loads of commonly detected pesticides in brood comb are associated with increased susceptibility of honey bees to the microsporidian parasite, *Nosema ceranae* (Wu et al., 2012), while exposure to the systemic neonicotinoid pesticide, imidacloprid, led to higher mortality and levels of *Nosema* sp. in the gut while simultaneously leading

to decreased nurse bee hypopharyngeal gland size (Alaux, et al., 2010b; Pettis et al., 2012). Exposure to these chemicals, additionally, may lead to a greater infection prevalence of some pathogens (e.g. *Nosema* spp.) that could then cause contamination and transmission of the pathogen at food collection sites and at the nest site (Vidau et al., 2011; Pettis et al. 2012; Wu et al., 2012).

Research is just recently beginning to uncover the difficult to identify and measure world of sub-lethal pesticide effects on honey bee colonies. Teasing apart the effects of pesticides is extremely difficult, particularly in a field setting, if outright death does not occur as a result of the exposure. Taking into consideration the interactions and potential synergies of multiple chemicals and routes of exposure becomes that much harder as a result.

1.6 Honey bee immunity (General overview)

Honey bees, as social insects, may be described as possessing multiple levels of immunity, including those at the colony (superorganismal), individual organismal, and genetic levels (Wilson-Rich et al., 2009). The various, sequential levels of immunity may be thought of as layers of an onion, beginning with the outermost layer represented by the exterior walls of the colony itself; each subsequent layer is more specific in its target than the previous one. As such, European (*A. mellifera*) and Asian (*A. cerana*, *A. koschevnikovi, A. nigrocincta*) honey bees are cavity-nesters; a characteristic providing a natural barrier limiting outside biotic (e.g. predation, parasitism) and abiotic (e.g. wind, precipitation, temperature) influences and fluctuations that would bring instability to the interior environment of the colony. This habit may be contrasted with the open-comb

nesting bees of Asia, including the giant honey bee (*A. dorsata*) and dwarf honey bees (*A. florea, A. andreniformis*).

At the entrance of the hive, adult "guard bees" discern nestmates from non-nestmates via olfactory cues, preventing entry to would-be intruders, be they individual honey bees from foreign colonies or invading predators and parasites. An additional layer of colony level immunity exists inside the hive as a resin (propolis) envelope on the walls around the brood area. Resins, secreted from plants and collected by foraging honey bees, provide an anti-microbial layer of protection inside the colony. This protection is particularly important toward minimizing the exposure of the relatively sensitive developing brood to bacterial and fungal pathogens such as *P. larvae* and *A. apis* (Simone et al., 2009).

Structural and behavioral barriers within the hive provide further layers of immunity to honey bee individuals and colonies. First, the waxy layer of the honey bee cuticle and the peritrophic matrix of the midgut provide physical barriers to pathogen invasion. Additionally, an acidic gut pH and the physical barrier provided by the gut epithelial cells create an added barrier to invasion. Furthermore, age structuring of bees within a colony (a phenomenon known as age polyethism) progresses in such a way as to minimize the exposure of the interior hive and brood to foreign invaders. Young, healthy adults are tasked with brood rearing, cell cleaning, queen attendance, and receiving/packing outside food resources into cells. As bees age, they assume progressively more risky roles: guarding the entrance, taking orientation flights, and finally foraging for resources. Auto- and allo-grooming of self and nestmates, respectively, aids in the removal of parasites, including *Varroa* and tracheal (*Acarapis*

woodi) mites (Evans and Spivak, 2010). Additionally, genetic traits such as hygienic behavior or *Varroa*-sensitive hygiene provide colonies with group level immunity to certain pathogens (*P. larvae, A. apis*) and parasites (*V. destructor*), and may be selected for by beekeepers (Wilson–Rich et al., 2009).

Finally, an individual bee's immune system is fine tuned to specific types of pathogen and parasite challenge and is composed of both the cellular and humoral arms of the immune system. Compared to other well-studied insect immune systems (e.g. *Anopheles gambiae, Drosophila melanogaster*) the honey bee possesses a reduced number of immune genes, including those involved in recognition, signaling, and response (Evans et al., 2006). This reduced number of immune genes in *A. mellifera* is likely due to the presence of a robust repertoire of social immunity mechanisms that has relaxed the need for such an extensive individual immune response over evolutionary time.

The cellular immune response is accomplished via freely circulating hemocytes that are tasked with recognizing and neutralizing large foreign bodies such as parasites and aggregations of bacteria in the blood. Hemocytes respond to pathogen associated molecular patterns (PAMPs) on the surfaces of bacteria and fungi, which are typically cell-wall components such as lipopolysaccharides, lipoteichoic acid, petidoglycans, or ß-1,3 glucans (Marmaras and Lampropoulou, 2009). Binding of hemocytes to pathogens leads to the aggregation, encapsulation, and hypoxia or starvation of the pathogen(s); a process often aided by the production and secretion of phenoloxidase by the hemocytes themselves (see below for further details).

In contrast, the humoral immune response is inducible, but also relies upon pattern recognition receptors present in the blood of honey bees, including proteins that recognize and bind to lipopolysaccharides and ß-1,3 glucans (Marmaras and Lampropoulou. 2009). Binding of such recognition proteins to pathogens in the hemolymph leads to cascades of intracellular protein activation and effector nuclear transcription and subsequent protein translation within the fat body. Depending on the class of PAMP present, several over-lapping pathways (Toll, IMD, JAK/STAT, JNK) may be triggered leading to the production of anti-microbial peptides, melanization, proliferation of hemocytes, and/or cell apoptosis (Tzou et al., 2002; Hoffmann, 2003; Evans et al., 2006; Marmaras and Lampropoulou. 2009; Gonzalez-Santoyo and Cordoba-Aguilar 2011).

Below is a further-detailed description of the immune response in individual insects, and honey bee when applicable and known. Immunity of individual honey bees is a relatively recently studied system because the superorganism has been long assumed to possess such a robust suite of mechanisms to preclude the rampant spread of diseases and parasites. Evidence such as the reduction in the diversity of honey bee immune genes, including effector genes and those involved in recognition and signaling, indeed suggests that the worker honey bee over evolutionary time has experienced relaxed selection for individual immunity in exchange for social immunity (Evans et al., 2006). However, myriad parasites and pathogens, particularly in the commercial beekeeping environment where bees from various regions come in contact with each other every year, continue to infect and infest bees, and new species continue to be discovered. Further, since diseases and parasites are transmitted bee to bee, leading to colony level

infections or infestations, it is worth a thorough examination of the mechanisms at work inside the cells of the superorganism (individual bees) as they fall victim or overcome such challenges.

1.6.1 Hemocyte-based immune response

Cellular immune defenses in insects are carried out by hemocytes. Insect hemocytes are responsible for several different types of cellular immune responses, including, phagocytosis, nodulation, encapsulation, clot formation around external wounds, and melanization (Lavine and Strand 2002; Marmaras and Lampropoulou 2009; Gonzalez-Santoyo and Cordoba-Aguilar 2011). Current understanding of hemocyte-mediated cellular immune defense is predominantly based on studies of *D. melanogaster*, select Lepidoptera, and mosquitoes (Strand 2008).

Phagocytosis involves the identification, engulfment, and intracellular destruction of foreign bodies and apoptotic cells by individual hemocytes (Marmaras and Lampropoulou 2009). Hemocytes may phagocytize bacteria, yeasts, and apoptotic cells in addition to synthetic structures such as nylon beads (Lavine and Strand 2002). The particular type of hemocyte responsible for phagocytosis varies across taxa, but typically involved are plasmatocytes and/or granulocytes, and possibly oenocytoids (Marmaras and Lampropoulou 2009).

Nodulation is a common cellular defense in insects that involves multiple hemocytes forming an overlapping sheath around a large number of bacteria in the hemocoel (Lavine and Strand, 2002). Similarly, encapsulation involves the activity of multiple hemocytes forming aggregations around a foreign object (Marmaras and Lampropoulou 2009), but this response is directed at larger targets, such as parasitoids,

nematodes, protozoa, and synthetic beads (Lavine and Strand 2002). The two most common hemocytes observed in nodule and capsule formation are granulocytes and plasmatocytes in Lepidoptera, and lamellocytes in *Drosophila* (Lavine and Strand 2002). Nodules and capsules do not always melanize, but in species where melanization does occur, oenocytoids and crystal cells (*Drosophila*) often play a role in the response (Lavine and Strand 2002). Melanization around the foreign object(s) leads to asphyxiation, and the production of oxygen and nitrogen free radicals that act to kill the invader (Marmaras and Lampropoulou 2009). Hemocytes also participate in the coagulation of hemolymph and clot formation around external wounds.

Studies examining the individual cellular immune responses of social insects typically measure total hemocytes, hemocyte concentration, and/or the encapsulation response. In *A. mellifera*, though colonies often vary widely, there is a trend toward decreasing numbers of circulating hemocytes, and hemocyte concentrations, as a bee ages (Schmid et al. 2005; Alaux et al.2010a; Alaux et al. 2010b). Additionally, cage studies have suggested that better protein may lead to a decreased concentration of hemocytes in circulating hemolymph (Alaux et al., 2010b).

1.6.2 The phenoloxidase-based immune response

The process of melanogenesis aids in the encapsulation of eukaryotic pathogens, the repairing of tissues, and in defense against bacteria (gram positive and negative), fungi, and even viral agents (Shelby and Popham 2006; Gonzalez-Santoyo and Cordoba-Aguilar 2011). Briefly, the amino acid, phenylalanine, is converted to tyrosine (by Phe hydroxylase), which is then converted to DOPA by activated phenoloxidase (PO)

(Gonzalez-Santoyo and Cordoba-Aguilar 2011). DOPA is then either converted to dopamine (facilitated by dopa decarboxylase), or is oxidized by PO to form dopaquinone (Marmaras and Lampropoulou 2009). Quinones produced in this way eventually polymerize and form melanin around a foreign body (Klowden 2007; Gonzalez-Santoyo and Cordoba-Aguilar 2011).

Melanin is deposited in nodules composed of aggregated hemocytes and microorganisms that form in the hemocoel of infected insects. Melanin is also frequently deposited as a coat during the cellular encapsulation response of insects to eukaryotic parasites, or after experimental injection of beads or other large foreign objects that promote encapsulation (Whitehorn et al. 2011). Melanized, nodulated or encapsulated foreign bodies cannot absorb nutrients, and are thus likely killed through starvation (Marmaras and Lampropoulou 2009; Gonzalez-Santoyo and Cordoba-Aguilar 2011). Additionally, cytotoxic reactive oxygen and nitrogen intermediates (reactive oxygen and nitrogen species) generated during the synthesis of melanin may aid in the killing of invading organisms (Marmaras and Lampropoulou 2009; Gonzalez-Santoyo and Cordoba-Aguilar 2011).

Costs of synthesis of PO and maintenance of the PO-producing system are thought to be high, thus, the system is thought to be highly "diet dependent" as evidenced in laboratory studies on the mealworm beetle, *Tenebrio molitor* (Siva-Jothy and Thompson 2002), the damselfly, *Hetaerina Americana* (Gonzalez-Tokman et al. 2011), the mormon cricket, *Anabrus simplex* (Srygley et al. 2009), and the caterpillars, *Spodoptera exemptra* and *S. littoralis* (Lee et al. 2006; Povey et al. 2009; Gonzalez-Santoyo and Cordoba-Aguilar 2011). The reason such a high cost is thought to be

associated with the PO system goes back to the fact that the primary compound involved in the proPO-activating system is tyrosine obtained from phenylalanine; an amino acid derived strictly from the diet. Additionally, the final product of the proPO-activating system is the nitrogen-rich compound, melanin, thus requiring substantial protein investment for the maintenance of the system (Lee et al., 2008).

The honey bee genome contains a single proPO gene (Evans et al. 2006), while two or more proPO genes have been identified in most other insects that have been investigated (Kanost and Gormon 2008), with *Aedes aegypti* possessing 10 (Waterhouse et al. 2007). Honey bee adult workers and queens exhibit an *increase* in measureable phenoloxidase with development and age, and unchallenged older individuals naturally exhibit higher levels of PO than unchallenged young individuals (Schmid et al. 2008; Wilson-Rich et al. 2008; Alaux et al. 2010a; Alaux et al. 2010b; Laughton et al. 2011) This is opposite to the trend observed in *Bombus terrestris* and *B. muscorum* (Moret and Schmid-Hempel 2009; Whitehorn et al. 2011). Drone honey bees, however, exhibit a decrease or no change in PO activity with age (Schmid et al. 2008; Laughton et al. 2011).

Genetics also seems to play a role in PO-based individual immunity in honey bees. Research has shown that increased colony genetic diversity leads to increased resistance to various pathogens (Tarpy 2003; Tarpy and Seeley 2006; Seeley and Tarpy 2007). Two separate studies found that PO activity varied significantly between colonies in the context of challenge with *Nosema* and/or imidacloprid and *A. mellifera* ontogeny paired with LPS challenge (Alaux et al. 2010b; Laughton et al. 2011).

With respect to the effects of diet or nutritional status on PO-based immunity, quality nutrition and a lack of parasites seems to positively affect the levels of PO

expressed in an individual honey bee worker. For example, Alaux et al. (2010a) found PO activity to be significantly higher in bees fed a high protein, polyfloral diet than in bees fed on a diet containing no protein. In another study, Alaux et al. (2011) found that *Varroa* mites seemed to inhibit the expression of proPO in parasitized adults as the proPO gene expression was greatest in unparasitized individuals regardless of whether they received pollen or not.

Studies on proPO and PO have also been conducted on immature honey bees. Evans et al. (2006) orally challenged 2nd instar larvae with *P. larvae* and observed an upregulation of the PPOact gene. Evans (2006), after visually examining 2nd instar larvae for the occurrence of *Paenibacillus larvae*, *Melissococcus pluton*, and *Ascophaera apis,* observed upregulation of the PPOact gene, but not the AmPPO gene, in those individuals with disease symptoms consistent with *P. larvae* and *A. apis*. As mentioned previously, Randolt et al. (2008) did not observe a change in PO activity in the hemolymph of 4th instar larvae 24 hours post-inoculation.

1.6.3 Humoral immunity and antimicrobial peptide (AMP) defense

Antimicrobial molecules responsible for the innate humoral immune response in insects, called antimicrobial peptides (AMP) are synthesized primarily in the fat body, although certain hemocytes may also produce them (Bulet et al. 1999; Klowden 2007). The activity of these peptides and polypeptides is thought to be quite broad, killing many strains of bacteria and fungi while having relatively low toxicity to the host organism (Hetru et al. 1998). Two nuclear factor signaling pathways control the expression of

AMPs upon immune challenge, the Toll and immune deficiency (Imd) pathways (Evans et al., 2006; Klowden 2007).

The Toll signaling pathway is generally thought to regulate the expression of AMP genes in response to invasion from gram-positive bacteria and fungi, while Imd regulates the gene expression of AMPs responding to gram-negative bacteria (Klowden 2007). Upon the induction of the immune response, AMPs are rapidly synthesized and released into the hemolymph where they persist for up to several days, depending on the species of insect (Hetru et al. 1998).

1.6.3.1 Defensin

Structurally, insect defensins contain a characteristic six cysteine/three disulfide bridge motif (Bulet et al. 1999), and these AMP's are mainly active against gram-positive bacteria (Hetru et al. 1998). Defensins are thought to act by disrupting the permeability of the bacterial membrane (Klowden 2007) that leads to a loss of cytoplasmic ATP and inhibition of respiration (Cociancich et al. 1993; Bulet et al. 1999).

In *A. mellifera* adults, due to the immuno-suppressive action of the *Varroa* mite, defensin1 has been shown to be expressed at higher levels in non-parasitized adult bees (Alaux et al. 2011). In another study, adult workers experienced elevated levels of defensin1 four days after being fed infective spores of *N. apis*, while no increase was observed upon infection with *N. ceranae* (Antunez et al. 2009). Defensin was also downregulated in response to *N. ceranae* infection in a separate study at 3 and 6 days post-infection (Chaimanee et al., 2012). Defensin 1 and 2 were both found to be highly upregulated in adult workers injected with *E. coli*, saline, or *P. larvae.*

Research has a ways to go in terms of a detailed and nuanced understanding of the immune response to varying pathogens. Accumulation of future studies will better clarify such variable immune responses with regard to different pathogens and parasite challenge on bees of varying health states, differently-aged bees, and genetic make-ups.

1.6.3.2 Abaecin

Antimicrobial peptides in this family contain a high proportion of proline and arginine and have been isolated from Hymenoptera, Diptera, and Hemiptera, (Hetru et al. 1998; Klowden 2007). Abaecin is most active against gram-negative bacteria, but instead of disrupting the bacterial membrane, these residues are thought to bind to bacterial proteins, thus taking longer than defensins to kill (6-12hr) (Klowden 2007).

Abaecin was found to be upregulated up to 4 days following challenge with *N. apis*, but was downregulated 7 days after *N. ceranae* challenge (no change at 4d) in adult *A. mellifera* workers (Antunez et al. 2009). Abaecin was also downregulated in response to *N. ceranae* infection in a separate study at 3 and 6 days post-infection (Chaimanee et al., 2012). Adults injected with *E. coli*, saline, or *P. larvae* showed increased levels of abaecin, while 2nd instar larvae showed no change after being injected with *P. larvae* (Evans et al. 2006). Evans and Pettis (2005) fed 1st instar larvae *P.l. larvae* and found that abaecin transcripts increased while colony disease and productivity decreased, with colonies showing the least disease symptoms exhibiting the highest abaecin levels.

1.6.3.3 Hymenoptaecin

Peptides in this family of AMPs tend to be larger and contain a higher proportion of glycine (Hetru et al. 1998; Klowden 2007). Glycine-rich peptides, including hymenoptaecin from honey bees, are primarily active against gram-negative bacteria. They are thought to increase the permeability of the outer and inner membrane of invading bacteria (Bulet et al. 1999; Klowden 2007).

In a study by Alaux et al. (2011), when adult honey bees were given pollen, hymenoptaecin gene transcripts were shown to increase, whether they were subjected to *Varroa* mites or not. However, non-*Varroa*-parasitized bees given pollen had higher expression of hymenoptaecin than those with mites and pollen (Alaux et al. 2011). Antunez et al. (2009) found hymenoptaecin to be upregulated in bees exposed to *N. apis*, but downregulated after exposure to *N. ceranae*. Hymenoptaecin was also downregulated in response to *N. ceranae* infection in a separate study at 3 and 6 days post-infection (Chaimanee et al., 2012). Hymenoptaecin increased in adult workers that were injected with *E. coli*, saline, or *P. larvae* (Evans et al. 2006). Adults injected with *E. coli* expressed higher hymenoptaecin at 24 hr p.i. (Randolt et al. 2008).

As described above, most challenge experiments have taken place in the lab in cages, and depend on many factors including the type of challenge, and the stage and age of bee exposed to the immune elicitor, and the immune response measured. As a result, the potential implications at the colony level are difficult to assess. Given the many potential parasites and pathogens that honey bees and colonies are faced with (see below), some of which have even been linked to CCD (Cox-Foster et al., 2007), future research should strive toward a better understanding of the relationship between parasite

and pathogen challenge, colony and individual bee health status, immune response, and ultimate colony outcomes.

1.6.4 Interactions between nutrition and immunity in honey bees

A handful of laboratory studies have examined the effects of pollen feeding on the nutritional and (to a limited degree) immunological systems of worker honey bees. Results indicate that compared to sugar syrup controls, and in the absence of immune challenge, high protein and pollen diets result in greater nutritional stores (including relative mass of the fat body, hypopharyngeal gland (HPG) size, and expression of the *vitellogenin* gene) (Alaux et al., 2010a; Alaux et al., 2011; Di Pasquale et al., 2013), a decreased cellular immune response, i.e. a quieter immune system (Alaux et al., 2010a), and decreased viral titers (DeGrandi-Hoffman et al., 2010).

Further, longevity after parasite challenge (e.g. with the microsporidian, *Nosema ceranae*) was enhanced when worker bees were fed either a high quality single source or mix of pollens compared to lower quality pollen and sugar syrup controls (Di Pasquale et al., 2013). In another study examining the interactions between nutrition and the acarine parasite, *Varroa destructor*, unparasitized (healthy, normal) worker bees fed pollen had higher Vg and lower hymenoptaecin expression than bees fed just sucrose solution, while *Varroa*-parasitized bees fed pollen expressed higher Vg and lower hymenoptaecin and apidaecin compared to parasitized bees fed just sucrose (Alaux et al., 2011). Bee challenged with lipopolysaccharide have been shown to exhibit decreased hypopharyngeal gland size (Alaux et al., 2012), while challenge with *N. ceranae* or *N.*

ceranae plus a systemic insecticide (imidacloprid) both led to significantly reduced HPG size (Alaux et al., 2010b).

These findings highlight just a few cases involving interactions between the nutrition and immune systems of the honey bee and yet leave a lot of unanswered questions, including whether these type of effects are manifested in the field.

1.7 Pests, parasites, pathogens

Pests, parasites, and pathogens are one of a handful of factors strongly influencing the health of honey bee colonies (Cox-Foster et al., 2007). Sampling and monitoring for honey bee pests, parasites, and diseases has become central to keeping bees, particularly over the last few decades. Certain parasites and pathogens may be more prevalent during certain times of the year (e.g. *Nosema* spp., viruses), exhibiting a natural ebb and flow as seasons change and in-hive dynamics adjust to altered conditions (Runckel et al., 2011). Other pests, such as *Varroa destructor* mites, increase their populations exponentially over the course of the summer, devastating colonies if not controlled culturally or chemically. Further, mites facilitate the spread of several honey bee viruses both within and between colonies (Martin et al., 2012).

Many honey bee pathogens infect the vulnerable developing immature larvae and pupae, including bacteria such as *Paenibacillus larvae* that causes American Foulbrood, *Mellissococcus plutonius* that causes European Foulbrood, the fungal pathogen, *Ascophaera apis* that causes Chalkbrood, and most of the honey bee viruses. Diseases and parasites may spread from weak and failing colonies to strong colonies inadvertently during "robbing" events when infective spores, found in various locations around the hive, are acquired by healthy foraging bees and brought back to their colonies of origin.

Additionally, contaminated flowers have potential to inoculate foraging bees with new pathogens or parasites left behind by sick individuals (Durrer and Schmid-Hempel, 1994; Colla et al., 2006; Singh et al., 2010).

1.7.1 *Nosema* spp.

Two species of microsporidia are known to infect honey bees, *Nosema apis* Zander (1909) and *Nosema ceranae* Fries (1996). Both species are obligate intracellular parasites, infecting the epithelial cells of the honey bee midgut (Larsson, 1986), however *N. ceranae* has additionally been detected in the tissues of the hypopharyngeal glands, salivary glands, malphigian tubules, and fat body (Chen et al., 2009). Once ingested, spores germinate in the lumen of the midgut of adult honey bees and rapidly produce more spores intracellularly (Bailey, 1955; Fries, 1988; Fries, 1989; Fries et al.,1992). *Nosema* spp. spores are likely acquired by young house bees when they remove fecal deposits, dead and diseased bees, and other foreign or contaminated material from the hive (Fries, 1997). Spores may also be ingested through contaminated food and at common water sources (Fries, 1997).

Spores of both species build up to extremely high levels in the lumen and within the midgut epithelial cells, and high levels of infection ultimately decrease the lifespans of infected bees (Fries, 1988; Fries et al., 1992; Higes et al., 2007a; Higes et al., 2007b; Paxton et al., 2007, Woyciechowski and Moron, 2009), Additionally, at the colony level, elevated *Nosema* sp. infection levels are correlated with higher supercedure rates and greater overwintering losses than those of uninfected colonies (Farrar, 1942).

Elevated infection levels may also severely interfere with nutrient absorption (Malone and Gatehouse, 1998) and ultrastructural alteration of cellular organelles (Wang and Moeller, 1971), leading to energetic stress and physiological and behavioral changes related to hunger, feeding, flight, and precocious foraging in honey bees (Mayack and Naug, 2009; Naug and Gibbs, 2009a; Kralj and Fuchs, 2010; Goblirsch et al., 2013). Foraging bees within a colony are known to contain the highest loads of *Nosema* sp. spores and, given their critical role in resource acquisition, this could affect the development and survival of colonies containing high levels of *Nosema* sp. spores (El-Shemy and Pickard, 1989; Smart and Sheppard, 2012).

Since 2007, *N. ceranae* has been found in populations of the western honey bee, *A. mellifera,* throughout much of the world including the U.S., Canada, South and Central America, Europe, North Africa, and Australia (Higes et al., 2006; Huang et al., 2007; Klee et al., 2007; Paxton et al., 2007; Calderon et al., 2008; Chen et al., 2008; Williams et al., 2008; Giersch et al., 2009; Higes et al., 2009). The current worldwide distribution of *N. ceranae* appears to be the result of a host switch or range expansion, although when this event occurred remains unclear (Chen et al., 2008; Klee et al., 2007; Paxton et al., 2007). However, evidence suggests that *N. ceranae* existed in U.S. managed honey bee populations as far back as the 1990s (Chen et al., 2008).

Cage studies have shown *N. ceranae* to be more virulent than *N. apis* (Higes et al., 2007a; Paxton et al., 2007) and Martin-Hernandez et al., (2007) found it to have a more sustained presence in the hive throughout the year, thus alarming beekeepers and scientists alike to the potential negative consequences of a new omnipresent microsporidian parasite of honey bees. Further, infection with *N. ceranae*, in contrast to

N. apis, leads to suppression of the humoral immune response (Antunez et al., 2009). The relatively recent detection of *N. ceranae* in populations of *A. mellifera* implicated it as a potential contributing factor to Colony Collapse Disorder (CCD) (Higes et al., 2007a; Martin-Hernandez et al., 2007; Cox-Foster et al., 2007).

1.7.2 *Varroa* spp.

The genus *Varroa* (Acari: Varroidae) is composed of several specialized species of obligate ectoparasitic mites that feed on the hemolymph of bees from the genus *Apis* (Anderson and Trueman, 2000). *Varroa jacobsoni* Oudemans (1904), originally infesting the Asian honey bee, *Apis cerana* (Koeniger, et al., 1981), underwent a host shift approximately around 1957, enabling it to parasitize the European honey bee, *Apis mellifera*, present alongside *Apis cerana* colonies in Asia. This expanded host range, coupled with the commercial movement of honey bee colonies, ultimately led to the nearly cosmopolitan distribution of *V. jacobsoni* (Anderson and Trueman, 2000). Further genetic analyses of *V. jacobsoni* demonstrated it to be a species complex composed of two sibling species: *V. jacobsoni* and *Varroa destructor* Anderson and Trueman (2000).

Varroa destructor is the most destructive pest of honey bee colonies worldwide, reproducing in brood cells and parasitizing both immature and adult bees (Ritter et al., 1984; Martin, 1994; Martin, 1995; Martin, 1998; Oldroyd, 1999; Martin, 2001; Romero-Vera and Otero-Colina, 2002; Guzman-Novoa et al., 2010). *Varroa destructor* levels tend to build through the summer when the queen is laying up to 2,000 eggs per day and ample brood is present to parasitize. Mated adult female mites may produce 4-6 offspring per cell and therefore colonies left untreated can build to high mite infestations

rapidly, killing or severely weakening bees going into winter (Amdam et al., 2004a). As a result, *V. destructor* mite infestation has been determined to be a primary, as well as interactive, cause of colony losses, and is particularly implicated in overwinter mortality (Dahle, 2010; Guzman-Novoa et al., 2010; LeConte et al., 2010).

Mites can affect the physiology of worker bees in infested colonies, causing weight loss, decreasing hemolymph carbohydrate and protein levels, including vitellogenin titers, and suppressing the immune response, including both humoral and cellular immunity (Bowen-Walker and Gunn, 2001; Amdam et al., 2004a; Gregory et al., 2005; Yang and Cox-Foster, 2005). Honey bees with depleted levels of vitellogenin have fewer protein reserves to access during the pollen-limited overwintering period inside the colony and, therefore, could reduce longevity and/or increase susceptibility to pathogens and parasites over the winter due to their suppressed immune system.

Furthermore, *V. destructor* is known to be associated with the prevalence of certain honey bee viruses (Nordstrom, 2003, Chen et al., 2004, Tentcheva et al., 2004; Shen et al, 2005a; Shen et al., 2005b; Chen et al., 2006) both through the direct transmission of viruses to brood and via the suppression of the honey bee immune system (Bowen-Walker et al., 1999; Yang and Cox-Foster, 2005). The presence of high *V. destructor* infestation levels potentially allows viruses and other microbes to replicate more readily in colonies highly infested with *V. destructor* (Yang and Cox-Foster, 2007).

Varroa destructor continues to be a main factor in honey bee colony losses, despite the continued production and use of many miticides to control the pest. Monitoring and sampling of colonies for *Varroa* mites is a must for any serious and sustainable beekeeping operation in the 21st century. Further, selection and breeding for

genetic traits, such as "hygienic behavior" (HYG) or "*Varroa*-sensative hygiene" (VSH), provide beekeepers an additional, non-chemical, tool in their toolkit for combating *Varroa* mites (Spivak and Gilliam, 1998; Spivak and Reuter, 2001; Ibrahim and Spivak, 2006). Bees that exhibit HYG traits are able to detect, uncap, and remove both *Varroa*-infested and diseased (*P. larvae, A. apis*) brood from the colony, while VSH bees exhibit more extreme hygienic behavior coupled with some degree of mite suppression, though the mechanism remains unknown. Selecting for such genetic traits that take advantage of the innate fastidiousness of certain bees lines to detect and remove infested pupae offer a more sustainable, long-term solution to the ongoing problem; one to which mites are unlikely to develop the resistance, or leave behind the harmful residues, associated with many commonly used, beekeeper-applied miticides.

1.7.3 Honey bee viruses

Approximately 18 viruses are known to infect honey bees, however six are most commonly detected infecting honey bees including, Deformed Wing Virus (DWV), Black Queen Cell Virus (BQCV), Sacbrood Virus (SBV), Kashmir Bee Virus (KBV), Acute Bee Paralysis Virus (ABPV), and Chronic Bee Paralysis Virus (CBPV) (Chen and Siede, 2007). Honey bee viruses are both horizontally and vertically transmitted and known to infect all castes and stages of honey bees; egg through adult, and workers, drones, and queens (Chen and Siede, 2007). Many of the viruses infecting honey bees remain largely latent in the bees, causing no overt disease symptoms. However, they may still dramatically affect the physiology and longevity of infected individuals, and may be triggered to replicate given appropriate stimuli such as *Varroa* mite feeding, immune

suppression, and/or poor nutrition (Martin, 2001; Chen et al., 2005; Chen and Siede, 2007; DeGrandi-Hoffman et al., 2010; Alaux et al., 2011; Runckel et al., 2011).

Deformed wing virus is the most prevalent of the honey bee viruses (Tentcheva et al., 2004; Berenyi et al., 2006; Chen and Siede, 2007), exhibiting an increased frequency of detection in honey bee colonies over the summer months, and reaching a maximum prevalence, concurrently with *V. destructor*, in the fall (Tentcheva et al., 2004; Chen and Siede, 2007). DWV is highly prevalent in the vector (*V. destructor*) (Topolska et al., 1995; Chatawannakul et al., 2006; Tentcheva et al., 2004; Martin et al., 2012) that acquires the virus from infected honey bees and then transmits the virus to new individuals upon subsequent feedings. *Varroa destructor*, in fact, has been putatively determined to be a competent vector of DWV in both laboratory and field settings (Bowen-Walker et al., 1999; Shen et al., 2005b), and further, feeding by *V. destructor* causes host immunosuppression and, as a result, increased DWV amplification (Yang and Cox-Foster, 2005). Phenotypically, the virus causes obvious symptoms in pupae and adults including shriveled, shrunken wings, small body size, and discoloration of infected adults upon eclosion (Ball and Bailey, 1997; Chen and Siede et al., 2007). Aside from these physical defects, infected individuals also exhibit physiological and behavioral abnormalities and experience premature death (Yang and Cox-Foster, 2006; Iqbal and Mueller, 2007; de Miranda and Genersch, 2010; Dainat et al., 2011).

Black Queen Cell Virus (BQCV) was first detected in dead immature queen honey bees that had turned dark brown and black inside their cells, hence the name (Bailey and Woods, 1977). The virus has been reported to be the most common cause of queen larval mortality in Australia (Anderson, 1993). Worker bees may also be infected

with the virus, but do not display overt disease symptoms (Chen and Siede, 2007). Queen bees are thought to become inoculated with the virus via contaminated brood food fed to them from infected nurse bees (Bailey, 1983). Infections with BQCV have bee reported worldwide (Ellis and Munn, 2005), and infection in worker bees seems to have a seasonal distribution, with increasing titers through the spring and summer months (Laidlaw, 1979; Runckel et al., 2011). Prevalence of the virus has been previously associated with increasing incidence of *Nosema* spp. in colonies (Bailey, 1981; Bailey, 1983). This finding is likely due to the mechanism by which *Nosema* infects the midgut cells of the honey bee (via harpooning of midgut epithelial cells), thus creating a path by which BQCV, and potentially other viruses, may enter the body of the honey bee (Chen and Siede, 2007). Black queen cell virus may be transmitted to honey bees via *Varroa* mite feeding, being detected in *Varroa* mites from Thailand (Chantawannakul et al., 2006), but not in France (Tentcheva et al., 2004). Further study is needed to elucidate the relationship between *Varroa* mites and BQCV transmission to honey bees.

Symptoms of Sacbrood virus (SBV) were first identified in the U.S. in 1913 and the virus has since been found on every continent where *Apis mellifera* is found (White, 1913; Ellis and Munn, 2005). Infected larvae initially turn pale yellow and subsequently their skin becomes leathery and larvae fail to pupate (Chen and Siede, 2007). Diseased larvae have the appearance of a water-filled sac when removed due to a large amount of virus-filled fluid accumulating between the body and skin (Chen and Siede, 2007). Dead larvae eventually become a dried, dark, brittle scale easily removed from the hive; a characteristic distinguishing SBV from the bacterial agent causing American Foulbrood, *Paenibacillus larvae*. Titres of SBV occur in both brood and adult stages of the honey

bee, but approximately 2-day old larvae are the most susceptible to the disease, while, as is the case with most honey bee viruses, adults remain asymptomatic (Ball and Bailey, 1997). Viral particles accumulate in the hypopharyngeal glands of nurse bees and are spread throughout the colony via feeding of brood and trophallaxis with adult nestmates. Additionally, infected foragers may inadvertently inoculate pollen loads with viral particles (Chen and Siede, 2007). As with BQCV, seasonality has been observed with SBV, with a greater prevalence in spring and summer (Tentcheva et al., 2004; Runckel et al., 2011). *Varroa* mites have not been putatively shown to vector SBV, however, the virus has been detected in *Varroa* mites and elevated SBV titres occurred in mite-infested honey bee colonies (Shen et al., 2005a; Antunez et al., 2006; Chantawannakul et al., 2006).

Kashmir Bee Virus (KBV) was first isolated in the 1970s from *Apis mellifera* in India and has subsequently been detected in honey bees in Australia, Oceania, Europe, and the U.S. (Bailey and Woods, 1977; Allen and Ball, 1995; Hung et al., 1996; Ellis and Munn, 2005). While no clearly defined disease symptoms exist with KBV (Anderson and Gibbs, 1988), the virus infects all life stages (Hornitzky, 1981) and is considered to be the most virulent of the honey bee viruses under laboratory conditions with mortality occurring in as few as three days after hemolymph inoculation with just a few viral particles (Chen and Siede, 2007). KBV does not, however, cause infection when spiked into food of adult bees (Chen and Siede, 2007). The occurrence of KBV is much lower than the previously discussed viruses and no clear seasonality seems to exist in honey bee colonies or apiaries (Tentecheva et al., 2004; Chen and Siede, 2007; Runckel et al., 2011). *Varroa* mites have been experimentally shown to effectively vector KBV via the

same mechanism as DWV transmission; the virus is acquired during blood-feeding on infected honey bees and transmitted to other individual bees (and mites) during subsequent feedings (Chen et al., 2004).

Acute Bee Paralysis Virus (ABPV) was initially discovered in the 1960s, causing paralysis and death in infected honey bees 5-6 days post-inoculation (Bailey et al., 1963). The virus exhibits an essentially worldwide distribution (Ellis and Munn, 2005), and seems to be more prevalent during the summer months (Runckel et al., 2011). Immature and adult stages may contain the virus, and despite the name, viral titers commonly occur in the field in apparently healthy bees (Bailey et al., 1981). Spread of the virus in a colony likely occurs via salivary gland secretions from infected adults to uninfected larvae during feeding, however ABPV has additionally been detected in *Varroa* mites that can act as vectors as well as activators of ABPV infection (Faucon et al., 1992; Chen and Siede, 2007).

Chronic Bee Paralysis Virus (CBPV), extracted from naturally-occurring paralyzed bees, was one of the first viruses isolated from honey bees (Bailey et al., 1968). The virus has been detected in adult bees from every continent on which honey bees are kept (Ellis and Munn, 2005; Antunez et al., 2006), exhibiting no distinct seasonality (Tentcheva et a., 2004). The virus causes two distinct phenotypic symptoms in adult honey bees, 1) trembling of the body and wings, crawling, bloating of the abdomen, and dislocation of the wings, and 2) hairless, shiny, and black bees (Bailey, 1975; Chen and Siede, 2007). CBPV does not replicate to disease-causing levels when introduced in food, however, topical application to denuded bees (hairs removed) resulted in positive infectivity (Bailey et al., 1983; Ball and Bailey, 1991). As a result, it is postulated that

conditions in the hive involving close quarters and/or decreased foraging activity could result in increased CBPV transmission (Chen and Siede, 2007). *Varroa* mites examined in France and Thailand were negative for CBPV, suggesting *Varroa* is an unlikely vector of the virus (Chen and Siede, 2007).

1.8 Conclusions

Clearly honey bees are confronted with multiple stressors, known and yet to be discovered, that interact in multiple ways within the individual bee and among colony members. We are just beginning to fit the pieces together with regard to the immune responses elicited in response to these various challenges, alone and in combination. This introduction summarizes what we know about bee health, from the larger landscape, effects on nutrition, colony-level measures of survival and pathogen/parasite loads, to individual levels measures of nutrition and immunity. My study is the first to attempt to link measures among all of the aforementioned levels into one cohesive narrative on the health of honey bee colonies.

To do so, I have compartmentalized the data from each level, and presented the level-specific data in each subsequent chapter. Chapter two addresses the question: How does land use contribute to colony productivity and survival? I analyzed the landscape data within a 3.2 km (2-mile) radius of each of six apiaries in the Prairie Pothole Region of North Dakota and quantified and categorized the dominant type of land use over three years. I then related land use to colony survivorship over three years. Chapter three addresses the question: What colony measures are predictive of colony health and survival? I analyzed the colony-level data gathered from regular (every six weeks) extensive assessments of 24 colonies positioned in each of the six North Dakota apiaries

(and California during the winter) over three years. Chapter 4 delves into data on individual worker honey bee physiology in the colonies positioned across the six study apiaries to identify measures of nutrition and immunity in response to land use and predictive of colony health and survival. Chapter five brings together the statistical modeling from all levels. Finally, in chapter 6, I describe the results of an experiment designed to test the nutritional and immunological effects of pollens collected in two of the six sites and fed to caged bees. This last study directly ties landscape effects to individual measures of bee health, and thus completes my characterization of the effects of land use on the health and survival of honey bees. Overall, this is the first study to demonstrate the critical importance of land use and floral resources in the landscape on colony survivorship , productivity, colony health, and individual bee relationship between nutritional physiology and immunity.

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1.10 Table Legend

Table 1.1 Trends in North Dakota crop acreage from1999 through 2013, and price per metric ton valuation, of important bee forage and commodity crops.

1.11 Table

Table 1.1

Crop	Year	Acreage ($x10^3$)	Price ($ per metric ton)	Price High ($ per metric ton)
Corn	1999	820	87.12	332.95 (Jul 2012)
	2013	3,850	207.41	
Soybeans	1999	1,350	180.64	622.91 (Aug 2012)
	2013	4,650	503.24	
Wheat	1999	9,410	113.19	439.72 (Mar 2008)
	2013	6,115	307.51	
Canola (oil)	1999	855	449.56	1,736.46 (Jul 2008)
	2013	920	985.02	
Sunflower (oil)	1999	1,700	464.32	2,300.19 (Jun 2008)
	2013	499	1,158.38	
Alfalfa (hay)	1999	1,450	79.80	219.00 (May 2013)
	2013	1,620	194.00	

CHAPTER 2

The influence of land use on the survival of commercial honey bee colonies in the Prairie Pothole Region of North Dakota, 2010-2013

Matthew Smart

2.1 Introduction

The managed European honey bee (*Apis mellifera*) is of critical importance for insuring the future availability and security of food in the United States and worldwide. In the U.S. alone, this single species of bee is responsible for the pollination of around 130 crops (McGregor, 1976), adding an estimated $15 billion annually to the value of honey bee-pollinated crops (Morse and Calderone, 2000). Worldwide, the total economic value of all pollinators is estimated to be around 194 billion dollars (Gallai et al., 2009) and recent declines in pollinator species include (e.g. *A. mellifera*) species of bees (e.g. *Bombus* spp.) as well as other native species of butterflies, bats, and birds, particularly those sensitive to land use and climate change, such as certain specialized and rare species, species at higher trophic levels, and cavity-nesting species (NRC, 2006; Burkle et al., 2013). The ability of beekeepers to inspect and manage *A. mellifera* colonies perhaps makes the occurrence of losses of this pollinator more visible than the losses incurred by other wild pollinators (NRC, 2006). Regardless, the phenomenon of sustained and severe annual losses of managed honey bee colonies (hobby, sideline, and commercial) has been mainly confined to North America and parts of Europe (NRC, 2006; vanEngelsdorp et al., 2008; Potts et al., 2010). More specifically, annual losses for commercial beekeepers (the subject of the present study) in the U.S. have hovered around 30% since 2006-07, with a low of 22.3% in 2011-12 and a high of 40.1% in 2012-13 (vanEnglesdorp et al., 2007; vanEnglesdorp et al., 2008; vanEnglesdorp et al., 2010;

vanEnglesdorp et al., 2011; vanEnglesdorp et al., 2012; Spleen et al., 2013; Steinhauer et al., 2014).

The upper-Midwestern states of North Dakota, South Dakota, Montana, and Minnesota have historically acted as an unofficial "bee refuge" for a large proportion of the managed, commercial honey bee colonies throughout the growing season. This region hosts around 1 million managed, commercial honey bee colonies from May-October every year, representing approximately 39% of the total U.S. managed, commercial pool of honey bee colonies (USDA, 2014b). Colonies transported to this region of the country for the summer by migratory beekeepers have done very well historically due, in large part, to an abundance of nectar and pollen-producing flowers present throughout the growing season. Critical regional blooms include perennial clovers and alfalfa (blooming Mid-July through September), canola (blooming early June), wildflowers (both native and non-native, including weeds), sunflower (blooming late July through August), and, more broadly, contributions from certain land use types such as livestock-grazed pastures, and more recently, conservation reserve program (CRP) lands (Gallant et al., 2014).

Steep declines in acreage of the above types of land use (alfalfa, canola, sunflower, CRP) have occurred across the Great Plains region over the last decade while concurrent acreage planted in non bee-utilized crops such as corn and soybeans has sharply increased (USDA, 2000; USDA, 2014a; Gallant et al., 2014). The expansion and increasing intensity of corn and soybean acreages brought on by relatively high commodity prices (Table 1.1) are particularly alarming given the large proportion of

beekeepers aggregating in the region each summer (USDA, 2000; USDA, 2014a; http://www.indexmundi.com).

Historically, the abundant bee forage in this region has been crucial for honey production and it has been assumed that good honey production in the summer months leads to success (better overwintering survival) of the colonies as they are transported to other regions for the winter months. Some migratory operations transport colonies to southern states where they are managed to produce new queen bees and new colonies ("package" bees and "nucs") for sale to other beekeepers and to replace any colony losses through the year. In more recent years, increasing numbers of colonies have been transported to California to pollinate a single crop, almonds.

Currently, approximately 1.5 million of the 2.5 million available colonies nationwide undertake the long journey to the central valleys (San Joaquin and Sacramento)of California. The 800,000 bearing acres of almonds in CA are 100% dependent on the pollination that they receive from honey bees. California is the only state in the U.S. producing almonds commercially and accounts for approximately 80% of the world's almond crop (Boriss and Brunke, 2005). Commercial cultivars of almond are self-incompatible, requiring the presence of rows of pollenizers within orchards. Flowering occurs from February-March each spring, and since daytime temperatures above approximately 57˚F are required for pollinating insect flight, the central California valleys are ideal locations for growing almonds. The 2012 almond crop totaled approximately 2 billion pounds and was valued at $4.3 billion (USDA, 2013). Beekeepers are compensated at a higher rate to pollinate almond compared to any other crop, particularly in recent years, earning around $140-160 for small colonies (6 frames

of bees) and $170-200 for larger colonies (8-10 frames) (Mader et al., 2010; Traynor, 2014).

With the recent and widespread change in land use in North Dakota, coupled with high colony losses and need for more, and more populous, colonies to pollinate almonds, attention has turned to how the landscape affects health and survival of honey bee colonies. Surprisingly, land use as an indicator of honey bee health and survival, and landscape-wide honey bee foraging patterns have been considered only in a few studies (e.g. Naug, 2009b; Couvillon et al., 2014). Most studies on honey bee foraging focus on honey production, pollination services, or foraging distances of colonies positioned around various crops and land use features (e.g., Beekman and Ratnieks, 2000; Steffan-Dewenter and Kuhn, 2003; Ricketts, 2004; Odoux et al., 2012; vanEngelsdorp et al., 2013; Gallant et al., 2014). Recent studies that have tracked survival of colonies in migratory beekeeping operations (e.g. Runckel et al., 2011; vanEngelsdorp et al., 2013) have not quantified the health and survival of colonies embedded in a migratory beekeeping operation in response to the specific landscapes to which the colonies are exposed.

This three-year study tested the degree to which land use around apiaries directly affected the annual survival of commercial honey bee colonies. The colonies began each year in North Dakota in May, and were transported to California in the fall where they remained until almond pollination in late February, and then were transported back to North Dakota, again, in May. The successes and failures and ecosystem services provided by honey bee colonies through pollination may be viewed in light of the overall quality of the surrounding lands in which colonies are placed by the beekeeper.

Specifically, I identified land use patterns within the larger agricultural matrix that were associated with higher vs. lower colony survival within six apiaries in North Dakota. This chapter represents the first step in statistical analysis that will be continued in subsequent chapters (3-5) examining how land use affected overall survival, colony-level and individual bee health in a commercial beekeeping operation.

2.2 Materials and methods

2.2.1 Treatment of colonies

One-hundred forty-four honey bee colonies owned and managed by Browning Bee Co. were positioned across six apiaries in the Prairie Pothole Region of North Dakota from 2010 through 2012 (24 colonies per site). Colonies were maintained in a typical U.S. commercial beekeeping configuration consisting of four colonies per pallet (therefore six pallets per apiary) facilitating transport of colonies on flatbed trucks. Each colony was tagged with a unique number above the entrance of the colony to facilitate quick identification of sampled hives. Colonies remained in North Dakota from May-September each year.

In October, pallets containing honey bee colonies were loaded onto flatbed trucks and shipped to California where the colonies were temporarily placed in "holding yards" consisting of open pasture land. Starting in mid-February, the colonies were positioned in almond orchards for pollination. Almond pollination is a significant economic source and motivation for many commercial beekeepers, and any impacts of spring through fall land use in North Dakota on annual colony survival should take into account the ability of colonies to survive and fulfill almond contracts previously arranged with growers.

Colonies were nutritionally supplemented as per typical commercial beekeeping practice with protein (to simulate pollen) and sugar syrup (to simulate nectar/honey). All colonies received Megabee® (Dadant and Sons) as their supplemental protein source twice in the spring (a single 1lb. patty in April and May) when no or few flowers were in bloom. As an experimental measure, half of the colonies received protein supplementation twice in the fall (the first half of the 24 colonies in each apiary received two-1lb. patties in August and September) to test if additional protein supplementation in fall would increase survivorship of colonies after transportation to California. Sugar syrup (1:1 w:v) was provided to colonies in spring and fall, with approximately 3.75 gallons given to each colony in each season.

Typical migratory beekeeping management practices were performed by the beekeeper on every colony to control pests and diseases. *Varroa destructor* mite populations were suppressed by laying a shop towel soaked in a 2:1 canola oil:Tactic® (10% amitraz) solution over the top bars of the top box each spring and fall (May and August-September after the honey harvest) for 14 days. *Nosema* spp. were controlled using Fumagilin-b® (Medivet Pharmaceuticals Ltd.) delivered in 1:1 sugar syrup twice per year in September and February. An antibiotic, Tylan® (Dadant and Sons), was used prophylactically to suppress bacterial infection, namely the causative agent of American Foulbrood, *Paenibacillus larvae*, and was delivered to the colony in sugar syrup twice per year with Fumagilin-b®. Finally, the beekeeper maintained a comb rotation scheme wherein one frame of foundation was inserted into, and one old frame removed from, each colony per year.

Colony inspections were carried out year-round with a minimum of six weeks occurring in between assessments. Inspections were conducted May-September in North Dakota, and November-March in California. Inspections consisted of assessing the adult and immature bee population sizes, colony pollen stores, queen status, and pest and disease levels (*V. destructor* mites, *Nosema* spp., virus levels, brood disease symptoms), and honey production. Honey production per site was determined by weighing all honey supers removed from each colony each fall and calculating the average weight (pounds) per site.

Agricultural pesticide exposure was sampled by trapping forager pollen loads coming into the colony. As forager bees returned to their colonies with pollen loads, each trap separated the pollen pellets from bee corbiculae and collected them into a drawer. Pollen was trapped multiple times over each summer in three sentinel colonies per site not involved in the larger experiment. Pollen samples were sent to USDA-AMS-National Science Laboratory in Gastonia, NC for analysis and residues (ppb) were reported back for 174 commonly used insecticides, fungicides, herbicides and metabolites. Pesticide data and the remaining colony data will be presented in detail in Chapter 3, wherein the colony level analyses occur. The result, however, showed no significant relationships between colony survival and pesticide exposure.

Survival of colonies at each site was determined as the proportion surviving from May of each year (when colonies were in North Dakota) through March of the following year (almond bloom in California). Colonies that died each year were replaced with new colonies before they returned to North Dakota each May. March was chosen as the survival cut-off point for determining survival because this was when the beekeeper made

a decision as to which colonies were suitable to be moved into almond orchards to fulfill pollination contracts. Additionally, most colonies that survive for use in almond pollination were healthy at the end of the almond bloom, so survival to almond bloom was a logical end point.

2.2.2 Land use assessments

Each year, land use in North Dakota was extensively surveyed on the ground within a 3.2 km (2.0 mile) radius of each of the six apiaries by a GIS technician from the USGS Northern Prairie Wildlife Research Center in Jamestown, ND. The technician visited each site multiple times each summer to document land use in the field on physical maps of the land surrounding each apiary, and data were entered into arc-GIS for final quantitative determination of the area occupied by each type of land use around each site in each year. Further, during each visit the technician assessed and estimated floral cover of various species of commonly occurring plants within each land use type at each site. Land use categories included: Conservation Reserve Program (CRP), fallow land, pasture land, hay land, flowering trees and shrubs, grassland, oil canola, oil sunflower, alfalfa, wetlands and cattails, shelterbelt, ditch, oats, wheat, corn, and soybeans (Table 2.1, Figure 2.2).

Due to the relatively low number of degrees of freedom available (6 sites x 3 years = 18 df), raw land use categories (Conservation Reserve Program (CRP), fallow land, pasture land, hay land, flowering trees and shrubs, grass land, oil canola, oil sunflower, alfalfa, wetlands, ditch, oats, wheat, corn, and soybeans) in North Dakota were combined into five groups, on which statistical analyses were carried out, including

1) Uncultivated potential bee forage (pasture, CRP, grassland, hayland, ditch, fallow land, flowering trees and shrubs), 2) Cultivated potential bee forage (alfalfa, canola, sunflower), 3) Wetlands, and 4) Non-forage (corn, soybeans, wheat, and oats).

2.2.3 Statistical analysis

Statistical analyses were carried out using R version 3.1.1 (R core team, 2014-07-10). Data were analyzed using lme4 (Bates et al., 2014) to perform a linear mixed effects analysis of the relationship between apiary survival (arc-sin square root-transformed) and (log-transformed) area of forage (m^2) within a 3.2-km radius of the 6 apiaries. As the fixed effect, area of uncultivated forage was entered into the model. Site and year were specified as random effects. Visual inspection of residual plots did not reveal any obvious deviations from homoscedasticity or normality. Diversity of land use (using the proportion of land use in each category at each site) was analyzed by calculating the Shannon-Weiner Diversity Index (H) for each site, defined as: $H = -\sum[(pi)*\ln(pi)]$, where pi in this case is the proportion of land use categories at a site relative to the total number of land use categories. A Jaccard similarity coefficient was also calculated to assess similarities between sites. Survival analysis was carried out using the library "survival" of R version 3.1.1.

2.3 Results

2.3.1 Relationship between land use, sites, honey, and apiary survival

There was a positive effect of the area (log-transformed) of uncultivated land in potential bee forage on annual proportional apiary survival (arc-sine square root

transformed) ($F_{1,16}$=12.4, r^2=0.44, p=0.003). Uncultivated bee forage included pasture, CRP, grassland, hayland, ditch, fallow land, and flowering trees and shrubs, while cultivated forage land included alfalfa, canola, and sunflower.

There were differences in the amounts (m^2) of land use in the 3.2-km area around each of the six sites (Table 2.1, Figures 2.2 and 2.3). Site 6 had the largest proportional area of uncultivated land, while site 3 had the most highly-cultivated area. The remaining sites had intermediate amounts of uncultivated land, relative to sites 3 and 6. The availability of floral resources around each apiary site varied (Table 2.2, Figure 2.3). The land use categories shown, CRP, pasture, hayland, ditch, and grassland contained the most floral resources as determined by on-the-ground surveys within the 3.2-km radius of each site, and thus represent the most likely targets for honey bee foraging. Within these categories, some areas were mowed (hayland, ditch) and others not (pasture, CRP, grassland).

Yearly land use change was quantified as an increase or decrease in the amount of land available from one year to the next that contained potential honey bee forage, which could greatly affect honey bee colony survival, honey production, and/or pollination economics. Over the three-year study, land use changed little in general (Table 2.3) with the greatest increase occurring in forage area from 2010 to 2011 around site 5 that experienced a nearly 2 million m^2 (22.45%) increase in floral resources from the previous year, due to a decline in the area planted in wheat and concurrent increases in hay and fallow lands. The greatest decline in bee forage area occurred at site 1 between 2010-2011 (1,263,720 m^2, 8.79% decrease in potential forage).

Based on the Shannon-Weiner diversity index, averaged across all three years, site 1 had the most diverse land use profile and site 3 the least (Figure 2.4), which is graphically evident in Figure 2.2. Jaccard similarity analysis resulted in sites 2 and 6 forming a cluster distinct from the other four sites (Figure 2.4). Sites 3 and 4 also had similar patterns of land use, as evidenced by their clustering.

Honey production interacted by site and year ($F_{10,414}$=7.1, p=2.8x10^{-10}, Table 2.5). Sites 1 and 6 generally had the greatest honey yields, while sites 2 and 3 generally produced the least. By year and across sites, honey production was greatest in 2012 and least in 2011.

2.3.2 Statistical model of the influence of land use on apiary survival

The land use categories were used as predictors of two separate response variables: average apiary honey yields and proportional apiary survival. Honey production and apiary survival were positively correlated ($F_{1,16}$=12.6, r^2=0.44, p=0.003). Land use categories included: area of uncultivated potential bee forage; area of cultivated potential bee forage; area of cultivated potential bee forage; and area of wetlands (see Table 2.1). Backward selection and Akaike's Information Criterion (AIC) were used to discern the model that best related the average apiary honey yields and annual apiary survival (arc-sine square-root transformed) to the land use profiles. The best model predicting honey yield, although not a statistically significant predictor, was the area (log-transformed) of uncultivated forage land ($F_{1,4.1}$ = 6.1, AIC = 161.42, p = 0.068). The best model predicting apiary survival was the area (log-transformed m^2) of uncultivated

forage in a 3.2-km radius around the apiaries ($F_{1, 4.3} = 14.31$, AIC = -22.69, p = 0.017) (Table 2.4).

2.3.3 Survival analysis

The proportion of surviving colonies was determined from May-March of each study year (Table 2.5). Colonies located at site 6 had the greatest survival every year with a high of 88% of colonies surviving in 2012, while site 3 exhibited the lowest survival of 50% in 2010 and 2011. The rate of colony mortality over the course of each of the three years (Figure 2.5 and Table 2.6) clearly showed that colonies in site 3 experienced more drastic declines compared to colonies in other sites, particularly in 2010-11 and 2011-12. Annual site colony survival was significantly different only between years 2010 and 2012 (2010-11: $X^2 = 11.5$, df=5, p =0.04; 2011-12: $X^2 = 10.9$, df=5, p =0.05) with colonies at site 3 experiencing the lowest annual survival. In 2012-13, survival was not significantly different among the six sites ($X^2 = 3.6$, df=5, p =0.61).

The greatest losses occurred from September through the following March (Figure 2.5). Of the colonies that died over the years, less than 10% appeared to die off suddenly: i.e. the colonies appeared to be healthy on one colony inspection but were dead by the following inspection six weeks later. From 9-31% of the colonies died by dwindling (or failure to thrive); i.e., the population of bees in a colony was observed to decrease over time until the colony died out. Only one incident of disease causing death was observed, in which the colony exhibited high levels of chalkbrood disease on the previous sample date. All other colony deaths were preceded by a problem with the queen bee (this will be discussed further in Chapter 3).

2.4 Discussion

In this portion of the study I determined the influence of land use on the honey production and survival of honey bee colonies in a migratory beekeeping operation. There was a significant impact of the amount (m^2) of uncultivated potential bee forage within a 3.2-km radius of honey bee colonies during the summer in North Dakota on the annual honey production and on the survival of those colonies during the winter months when they were in California for almond pollination. The summer location with the greatest annual survival, site 6, possessed the greatest area in potential bee forage (~70% of the surrounding land within a 3.2 km radius) over all three years.

A large component of land use surrounding site 6 was pasture, where many of the weedy and non-native species preferred by honey bees were detected on the ground (Table 2.2). In contrast, site 3, which had no nearby land in pasture, experienced the lowest annual survival (in 2010 and 2011, 2nd lowest in 2012). Site 3 was determined to be the least diverse in terms of overall land use, and further, possessed the least amount of potential bee forage (~12% of the total area), most of which was in CRP with far less bee forage compared to site 6 (Table 2.2). Site 1 had the highest diversity of land use, graphically depicted in Figure 2.2, though still maintained more total area in non-bee forage compared to sites 2 and 6.

The 12-17% annual mortality at site 6 fell within the "acceptable range" of annual losses that most beekeepers would historically expect to experience (vanEngelsdorp et al., 2008; Ellis et al., 2010), and was much closer to annual losses experienced by beekeepers prior to the spread and establishment of the *V. destructor* mite to the U.S in the 1980s (vanEngelsdorp, pers. comm.). Mite and other parasite and pathogen levels in

this study were not materially different across sites (see Chapter 3). This finding suggests that the abundance, density and/or quality of flowers available to bees for nutrition during the growing season more strongly impacted overall honey bee health and long-term colony survival compared to the effects of parasites or diseases. Therefore, identification and selection of quality sites containing forbs that bloom over the growing season in a 3.2-km radius around an apiary (assuming they are present in a given landscape) is critical for successful beekeeping in a shrinking pool of potential forage.

Sites 3 and 6 represented the locations with the greatest degree of divergence in survival and land use, while the remaining four sites fell in between. It is important to note that there likely exists a lower threshold for available bee forage around an apiary below which the landscape can no longer support a given number of colonies. It is possible that had fewer colonies been placed at site 3, for example, there may have been greater survival at that site. Commercial beekeepers with 10,000+ colonies such as the collaborator in this study, require sites that can sustain a large number of hives as it becomes spatially, temporally, and economically untenable to manage a much larger number of apiaries composed of fewer colonies.

Statistical analysis indicated that the total area (m^2) of land use, rather than the proportion of the total within a 3.2-km radius, was the most significant predictor of both honey production and annual apiary survival. This is a critical practical point for beekeepers attempting to choose quality, productive sites for their colonies. Balance and diversity of land use are important factors (and unavoidable in the real world). However, the available potential bee forage must exist above a certain quantitative threshold in the surrounding landscape to support and sustain colonies over the growing season.

Importantly, areas in CRP land were roughly equal at sites 3 and 6, however, on-the-ground surveying showed that a far greater abundance of flowering plants occurred in the CRP around site 6 (Table 2.2), specifically in sweet clover (*Melilotus* spp.) and alfalfa (*Medicago sativa*). Such differences could be due to several factors including differences in seed mixes, weed and land management, and differences resulting from soil nutrients and water availability. As such, care should be taken in evaluating the impacts of such programs with respect to their benefits to honey bee colony health and survival. Seed mixes should be chosen that are maximally beneficial to pollinators (and maintained to protect continued growth of forbs so they are not outcompeted by grasses) if the goal is to significantly increase pollinator forage on the landscape.

Site 2 was located inside the Arrowwood National Wildlife Refuge, approximately 75,000 acres of U.S. Fish and Wildlife Service lands composed of a large amount of grassland with native and non-native forbs distributed throughout refuge. Colonies were positioned in an area near the middle-eastern edge of the refuge such that they had access to Fish and Wildlife lands to the west and were susceptible to agriculturally managed private lands to the east outside the refuge. This was the first time honey bee colonies were allowed access to this site managed primarily for local and migratory birds. Floral data suggested that over the three-year study period, land cover specifically in *Melilotus* spp., *Grindelia squarrosa*, and *Sonchus* spp. increased along with honey production and survival, while other types of floral cover such as alfalfa, native sunflower, and goldenrod remained relatively unchanged in the grassland. Survey data showed that bees in the refuge had relatively little alternative potential forage to that

occurring in the grassland (though some alfalfa hayland was nearby) with very little land in pasture, CRP, fallow land, or ditch occurring near the site.

Recent data shows that the area of potential bee forage is on the decline in the Great Plains region, being replaced with row crops of little value to bees (USDA, 2014a; Gallant et al., 2014). This trend is particularly alarming given the importance of the region for summering of commercial, migratory honey bee colonies critical for U.S. honey production, pollination of fruits, nuts, berries, and vegetables across the country, and production of queens and "package bees" sold to beekeepers in the spring to make up for winter losses. North Dakota leads the nation annually in honey production and has historically been a plentiful summering forage (nectar and pollen) location for bees, due largely to the presence of perennial and biennial volunteer "weeds" such as clovers (*Melilotus* spp., *Trifolium* spp.), thistles (*Circium* spp., *Sonchus* spp.), goldenrod (*Solidago* spp.), vetch (*Vicia* spp.), gumweed (*Grindella squarrosa*), native non-cultivated sunflowers (*Helianthus* sp.), and dandelion (*Taraxacum officinale)*, in addition to cultivated varieties of canola (*Brassica napus*), sunflower (*Helianthus annuus*), and alfalfa (*Medicago sativa*). However, there is an alarming shift in the way land is managed in North Dakota. Gallant et al. (2014) showed that the area planted in such cultivated varieties of potential bee forage have been in decline on the North Dakota landscape over the past decade, while area planted in corn and soybeans has greatly increased. In fact, both weeds and cultivated forage crops are facing declines across the region due to a number of factors including relatively high recent commodity crop prices, intensive and varied (by state and county) mowing regimes in ditches and roadsides, and exclusion from glyphosate resistant crop fields and borders (e.g. soybeans). These, and

other factors, are combining to severely limit the spatial and temporal availability and patterns of potential bee forage that are required en masse across the entire growing season to support the approximately 1 million honey bee colonies present each year in the Upper Midwestern Region.

As the area of bee forage declines, beekeepers must compensate by feeding their colonies. Significant economic inputs go into the nutritional supplementation and pest suppression of honey bee colonies, while outputs include honey production, fulfilling almond pollination contracts, and (in other operations than the one studied here) selling package bees and queens. For example, each spring and fall colonies typically receive protein supplementation and sugar syrup to stimulate colony growth and to increase food stores for survival and brood production. Likewise, each spring and fall colonies receive chemical treatments to control *V. destructor* mites and a fungal parasite, *Nosema* spp., and antibiotics to suppress bacterial pathogens. The greatest number of colony losses occurred incrementally between September-March: i.e., winter losses occurring prior to almond pollination. Colonies such as these, particularly from sites with low apiary survival that do not make it to almond pollination, result in economic losses at multiple levels in the forms of wasted nutritional supplements and chemical controls, reduced or absent honey production, unfulfilled almond pollination compensation, increased fuel and labor costs in cross-country shipping of colonies that will not survive winter, and the costs associated with the splitting of existing colonies and purchasing of new colonies to make up losses every year. Such costs associated with failing and dead colonies represent a substantial burden on commercial beekeepers, creating a large degree of uncertainty in business solvency from year to year.

The limited number of sites (6) and years (3) in the current study characterize a relatively narrow, yet representative (in terms of land use a typical beekeeper might encounter in the region of interest), group of data points. Future studies including many more sites and diverse types of land use will likely better inform the relationship between land use and honey bee colony survival to a much finer degree, enabling researchers to hone in on more micro-scale landscape effects, including cover types such as crop borders, restored prairies, alternative CRP seed mixes, organic farms, cover crops, etc. Such research will result in greater resolution for beekeepers, thus affording them the ability to conduct "precision beekeeping" with respect to site selection and expected outputs based on land use.

Beekeepers depend on healthy bees for their livelihoods. Healthy bees make honey and fulfill almond pollination contracts, however, the distinction between healthy and unhealthy colonies is often not realized until after all colonies have received costly inputs (feeding, medications) with no guarantee of productivity by the end of the summer or survival through the winter. Here I have shown that a strategic selection of apiary sites by a beekeeper has value on predicting productivity and survival. Therefore site selection is one critical factor that beekeepers, importantly, have control over to improve the productivity and survival of colonies in their operations.

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2.6 Table legends

Table 2.1 Total (m^2) and proportional land use area around the six study apiaries over the three-year experimental period, 2010-2012. Categories of potential bee forage included 1) Uncultivated potential bee forage (pasture, CRP, grassland, fallow land, flowering trees and shrubs, hayland, ditch), 2) Cultivated bee forage (canola, sunflower, alfalfa), and 3) wetlands. Non-forage included corn, soybeans, wheat, and oats.

Table 2.2 Ground survey estimates of floral cover area ($m^2 \times 10^3$) within land use categories (averaged over three summers, 2010-12). Each year land within a 3.2 km (2-mile) radius was extensively surveyed for presence of floral resources. Total flower species cover within each land use category was averaged across three years. Proportion of flower cover for a given type of land use was defined as the total floral cover divided by the mean area of that land use for a given site.

Table 2.3 Land use change across the six study apiaries 2010-2012. Both total m^2 and percent change for each site and year are shown relative to the first year of the study (2010). Forage land use categories included CRP, fallow land, pasture, hay land, flowering trees and shrubs, grassland, canola, sunflower, alfalfa, wetlands, shelterbelt, cattails, and ditch. Non-forage included lands planted in oats, wheat, corn, and soybeans.

Table 2.4 Linear mixed effect models of annual proportion of colonies surviving and honey yields. Site and year were partitioned as random effects using R package lme4.

Table 2.5 Proportion of surviving colonies and honey production by site and year, 2010-2013. Each site was composed of 24 colonies beginning in May of each year (in North Dakota apiaries). Final survival was determined in March of the following year (in California almond orchards). Honey production is reported as mean pounds per colony per site and year.

Table 2.6 Annual χ^2 apiary survival analysis, 2010-2012. Number of observed and expected colony deaths at each site and year are depicted in columns three and four. Degrees of freedom ((n-1) from 6 sites per year), χ^2 values, and p-values associated with the significance of survival are depicted in the final three columns.

2.7 Tables

Table 2.1

Land use	Area (m^2 x10^3)/ Prop.	Site / Year 2010	1 2011	1 2012	2 2010	2 2011	2 2012	3 2010	3 2011	3 2012	4 2010	4 2011	4 2012	5 2010	5 2011	5 2012	6 2010	6 2011	6 2012
CRP	m^2	5603	4849	3641	650	650	650	3238	2986	2986	433	398	433	304	555	199	3209	3209	3209
	prop.	0.174	0.150	0.113	0.020	0.020	0.020	0.100	0.092	0.092	0.013	0.012	0.013	0.009	0.017	0.006	0.099	0.099	0.099
Fallow	m^2	-	-	-	-	1279	61	-	-	-	-	2524	-	-	1706	-	757	10	522
	prop.	-	-	-	-	0.039	0.002	-	-	-	-	0.078	-	-	0.053	-	0.023	0.000	0.016
Pasture	m^2	2861	2467	2433	2127	2127	3377	-	-	-	1150	1150	1150	5221	4459	5194	14714	14161	14719
	prop.	0.089	0.076	0.075	0.066	0.066	0.104	-	-	-	0.036	0.036	0.036	0.163	0.139	0.162	0.455	0.438	0.455
Hayland	m^2	898	807	1375	1617	154	1223	151	173	37	1976	1966	1976	942	1279	634	2468	2116	2478
	prop.	0.028	0.025	0.043	0.050	0.005	0.038	0.005	0.005	0.001	0.061	0.061	0.061	0.029	0.040	0.020	0.076	0.065	0.077
Flowering trees and shrubs	m^2	538	538	538	625	625	625	142	142	142	188	188	188	705	705	705	478	478	478
	prop.	0.017	0.017	0.017	0.019	0.019	0.019	0.004	0.004	0.004	0.006	0.006	0.006	0.022	0.022	0.022	0.015	0.015	0.015
Grassland	m^2	138	705	39	11634	12078	9942	108	108	108	79	79	79	1269	1269	1098	621	1432	621
	prop.	0.004	0.022	0.001	0.359	0.372	0.306	0.003	0.003	0.003	0.002	0.002	0.002	0.040	0.040	0.034	0.019	0.044	0.019
Canola	m^2	1467	874	1019	-	-	-	-	-	-	-	-	-	-	-	-	-	59	-
	prop.	0.046	0.027	0.032	-	-	-	-	-	-	-	-	-	-	-	-	-	0.002	-
Oil Sun-flower	m^2	-	-	1414	-	-	-	-	26	-	563	-	-	-	-	-	-	-	-
	prop.	-	-	0.044	-	-	-	-	0.001	-	0.017	-	-	-	-	-	-	-	-
Alfalfa Hayland	m^2	-	-	-	-	363	125	-	-	-	-	-	-	122	501	199	27	773	162
	prop.	-	-	-	-	0.011	0.004	-	-	-	-	-	-	0.004	0.016	0.006	0.001	0.022	0.005
Wetlands	m^2	382	382	409	4853	4853	4852	3155	3175	3207	1227	1227	1187	4357	4357	4357	2181	2264	2181
	prop.	0.012	0.012	0.013	0.150	0.150	0.150	0.097	0.098	0.099	0.038	0.038	0.037	0.136	0.136	0.136	0.067	0.070	0.067
Shelter-	m^2	350	350	350	89	89	28	160	160	160	115	115	115	131	131	132	158	158	158

		Site	1		2			3			4			5			6		
Land use	**Area (m^2 x10^3)/ Prop.**	**Year 2010**	**2011**	**2012**	**2010**	**2011**	**2012**	**2010**	**2011**	**2012**	**2010**	**2011**	**2012**	**2010**	**2011**	**2012**	**2010**	**2011**	**2012**
belt	prop.	0.011	0.011	0.011	0.003	0.003	0.001	0.005	0.005	0.005	0.004	0.004	0.004	0.004	0.004	0.004	0.005	0.005	0.005
Cattails	m^2	1584	1584	1933	734	734	734	811	811	759	2080	2080	2081	216	216	216	1626	1626	1583
	prop.	0.049	0.049	0.060	0.023	0.023	0.023	0.025	0.025	0.023	0.064	0.064	0.064	0.007	0.007	0.007	0.050	0.050	0.049
Ditch	m^2	554	556	553	219	219	219	356	356	356	718	717	718	534	534	531	487	503	487
	prop.	0.017	0.017	0.017	0.007	0.007	0.007	0.011	0.011	0.011	0.022	0.022	0.022	0.017	0.017	0.017	0.015	0.016	0.015
Oats	m^2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	130	129	218
	prop.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.004	0.004	0.007
Wheat	m^2	3900	4097	1997	2170	3373	400	4231	5655	2493	5687	4877	4790	7717	4697	5524	1038	1431	1328
	prop.	0.121	0.127	0.062	0.067	0.104	0.012	0.131	0.175	0.077	0.176	0.151	0.148	0.241	0.147	0.173	0.032	0.044	0.041
Corn	m^2	4249	4738	6380	1542	1557	4044	4974	4164	8264	5755	4109	7177	1015	1605	1865	1412	858	1897
	prop.	0.132	0.147	0.198	0.048	0.048	0.125	0.154	0.129	0.255	0.178	0.127	0.222	0.032	0.050	0.058	0.044	0.026	0.059
Soybeans	m^2	9723	10302	10154	6186	4345	6167	15034	14604	13848	12288	12829	12366	9448	9966	11325	3053	3214	2327
	prop.	0.302	0.319	0.315	0.191	0.134	0.190	0.465	0.451	0.428	0.381	0.398	0.383	0.295	0.312	0.354	0.094	0.099	0.072
Total	m^2	32247	32248	32235	32445	32446	32446	32359	32359	32359	32258	32258	32260	31981	31981	31978	32358	32362	32368
Bee Forage: Un-cultivated	m^2	10592	9922	8579	16873	17132	16097	3995	3765	3629	4544	7022	4545	8974	10507	8361	22734	21909	22514
Bee Forage: Cultivated	m^2	1467	874	2433	-	363	125	-	26	-	563	-	-	122	501	199	27	773	162
Sum bee forage	m^2	12059	10796	11012	16873	17495	16222	3995	3790	3629	5107	7022	4545	9097	11009	8560	22761	22682	22676
Prop. bee forage	prop.	0.374	0.335	0.342	0.520	0.539	0.500	0.123	0.117	0.112	0.158	0.218	0.141	0.284	0.344	0.268	0.703	0.701	0.701

Table 2.2

Land use	Site	Sweet Clover	Alfalfa	Gum-weed	Native Sunflower	Sow-thistle	Golden-rod	Total	Mean area (m^2x10^3) site land use	Proportion flower cover
CRP	1	154	297	54	403	182	427	1519	4698	0.32
	2	-	325	-	-	-	-	325	650	0.50
	3	341	19	45	96	191	42	734	3070	0.24
	4	107	21	21	22	-	64	237	421	0.56
	5	48	138	12	24	-	12	233	353	0.66
	6	650	1206	39	162	162	160	2379	3209	0.74
Ditch	1	100	49	49	41	16	43	298	555	0.54
	2	7	19	13	10	3	11	63	219	0.29
	3	60	28	57	20	39	20	225	356	0.63
	4	94	68	19	117	19	117	434	718	0.60
	5	-	105	-	-	-	-	105	533	0.20
	6	87	36	36	28	14	78	279	492	0.57
Grassland	1	10	10	-	10	10	10	52	294	0.18
	2	551	240	212	139	111	557	1809	11218	0.16
	3	-	-	-	-	-	8	8	108	0.07
	4	-	-	-	-	-	-	-	79	-
	5	-	-	-	-	-	-	-	1212	-
	6	109	27	27	41	27	97	328	891	0.37
Hayland	1	71	602	32	68	32	25	830	1027	0.81
	2	-	869	11	-	-	-	880	998	0.88
	3	5	15	-	-	-	-	19	121	0.16
	4	68	85	77	17	9	-	256	1973	0.13
	5	181	416	-	-	19	-	615	951	0.65
	6	348	1151	24	18	18	-	1558	2354	0.66
Pasture	1	-	38	213	87	-	234	573	2587	0.22
	2	29	7	35	7	1	42	121	2544	0.05
	3	-	-	-	-	-	-	-	-	-
	4	77	95	176	-	-	124	472	1150	0.41
	5	-	2	22	-	-	-	24	4958	0.005
	6	3554	187	1624	1146	608	2391	9885	14531	0.68

Table 2.3

m^2 change from 2010		Site					
Year	**Land use**	**1**	**2**	**3**	**4**	**5**	**6**
2011	Area forage	-1,263,720	622,374	-184,036	1,914,928	1,912,047	4,279
2011	Area non-forage	1,265,098	-621,863	184,036	-1,915,006	-1,912,054	-694
2012	Area forage	-671,231	-712,104	-366,004	-600,979	-536,428	-128,575
2012	Area non-forage	659,454	713,051	366,004	602,549	533,912	138,136

Percent Change from 2010		Site					
Year	**Land use**	**1**	**2**	**3**	**4**	**5**	**6**
2011	Area forage	-8.79%	2.76%	-2.27%	22.45%	13.85%	0.02%
2011	Area non-forage	7.08%	-6.28%	0.76%	-8.07%	-10.52%	-0.01%
2012	Area forage	-4.67%	-3.16%	-4.51%	-7.05%	-3.89%	-0.48%
2012	Area non-forage	3.69%	7.20%	1.51%	2.54%	2.94%	2.45%

Table 2.4

Model (Survival)	**Effect**	**DF**	**Value**	**SE**	**T**	**P**
$\sin^{-1}(\sqrt{(\text{prop. survival})})$ ~ log(area uncultivated forage)	Intercept	4.26	-0.938	0.64	-1.46	0.21
	Log(Area uncultivated forage)	4.26	0.123	0.04	3.06	0.031*
Random Effects	**Intercept**		**Variance**	**S.D.**		
	Site		0.002	0.05		
	Year		0.0001	0.01		
	Residual		0.005	0.07		

Model (Honey)	**Effect**	**DF**	**Value**	**SE**	**T**	**P**
(honey ~ log(area uncultivated forage)	Intercept	4.11	-291.91	150.7	-1.94	0.123
	Log(Area uncultivated forage)	4.06	21.43	8.67	2.47	0.068
Random Effects	**Intercept**		**Variance**	**S.D.**		
	Site		11.33	3.4		
	Year		441.77	21.0		
	Residual		431.26	20.8		

Table 2.5

Site	Year	Survival (Proportion)	Honey production (lbs.)
1	2010	0.79	101.04
	2011	0.75	59.18
	2012	0.71	115.09
2	2010	0.71	59.38
	2011	0.71	39.74
	2012	0.79	110.34
3	2010	0.50	27.40
	2011	0.50	38.48
	2012	0.71	98.13
4	2010	0.75	73.96
	2011	0.75	88.59
	2012	0.67	75.75
5	2010	0.83	67.29
	2011	0.75	81.06
	2012	0.75	79.17
6	2010	0.83	103.65
	2011	0.83	63.56
	2012	0.88	140.16

Table 2.6

Year	Site	Observed	Expected	(O-E)^2/E	(O-E)^2/V	DF	X^2	P-value
2010	1	5	6.60	0.3885	0.5108	5	11.5	0.04*
	2	8	6.59	0.3029	0.3985			
	3	12	5.61	7.2785	9.2909			
	4	6	6.30	0.0143	0.0186			
	5	4	6.92	1.2309	1.6355			
	6	4	6.98	1.2743	1.6975			
2011	1	6	6.74	0.0813	0.1060	5	10.9	0.05*
	2	7	7.53	0.0375	0.0501			
	3	12	5.41	8.0351	10.0724			
	4	6	7.05	0.1573	0.2069			
	5	6	6.78	0.0888	0.1158			
	6	4	7.49	1.6270	2.1714			
2012	1	7	6.35	0.06650	0.08696	5	3.6	0.61
	2	5	5.71	0.08804	0.11244			
	3	7	5.58	0.35867	0.45703			
	4	8	5.67	0.95891	1.22299			
	5	6	6.20	0.00642	0.00835			
	6	3	6.49	1.87525	2.46363			

2.8 Figure legends

Figure 2.1 Linear regression and line-of-best-fit of log transformed area of bee forage on annual apiary survival (arcsin square root-transformed). Survival of 24 colonies at each of six sites was recorded over three years.

Figure 2.2 Characterization of land use area (m^2) within a 3.2 km (2.0 mi.) radius by site (averaged over three summers), 2010-2012. Apiary sites consisted of 24 colonies that were located in the Prairie Pothole Region of North Dakota. Order of figure legend on the right corresponds with the order of land use in the column graphs.

Figure 2.3 Proportion of bee forage area (m^2) within 3.2-km radius of each apiary in the summer, 2010-2012. Pie charts depict the overall land use at each site and year. Five categories are depicted within each pie chart, and starting at the 12 o'clock position: 1) (dark green) the proportion of uncultivated potential bee forage land in flowers (CRP, pasture, fallow, grassland, hayland, ditch), 2) (light green) the proportion of uncultivated potential bee forage land with no flowers, 3) (orange) the proportion of cultivated potential bee forage land in flowers (canola, sunflower, alfalfa, hayland, ditch), 4) (blue) the proportion of wetlands, and 5) (grey) the proportion of non-forage (corn, soybeans, wheat, oats). Each site consisted of 24 honey bee colonies.

Figure 2.4 Shannon-Weiner diversity index and Jaccard similarity tree for land use across the six study sites and 3 years, 2010-2012.

Figure 2.5 Survival of colonies across the six North Dakota study sites, 2010-2012. Colonies were assessed and proportion surviving tallied at each site every six weeks year-round, May – March.

2.9 Figures

Figure 2.1

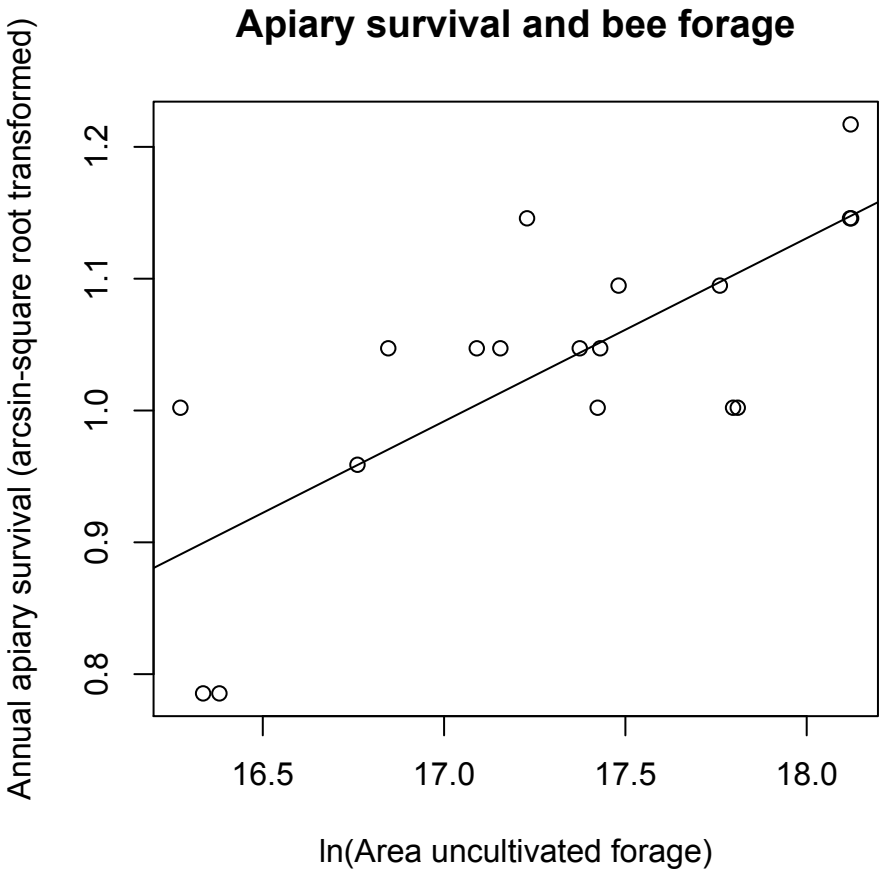


Figure 2.2

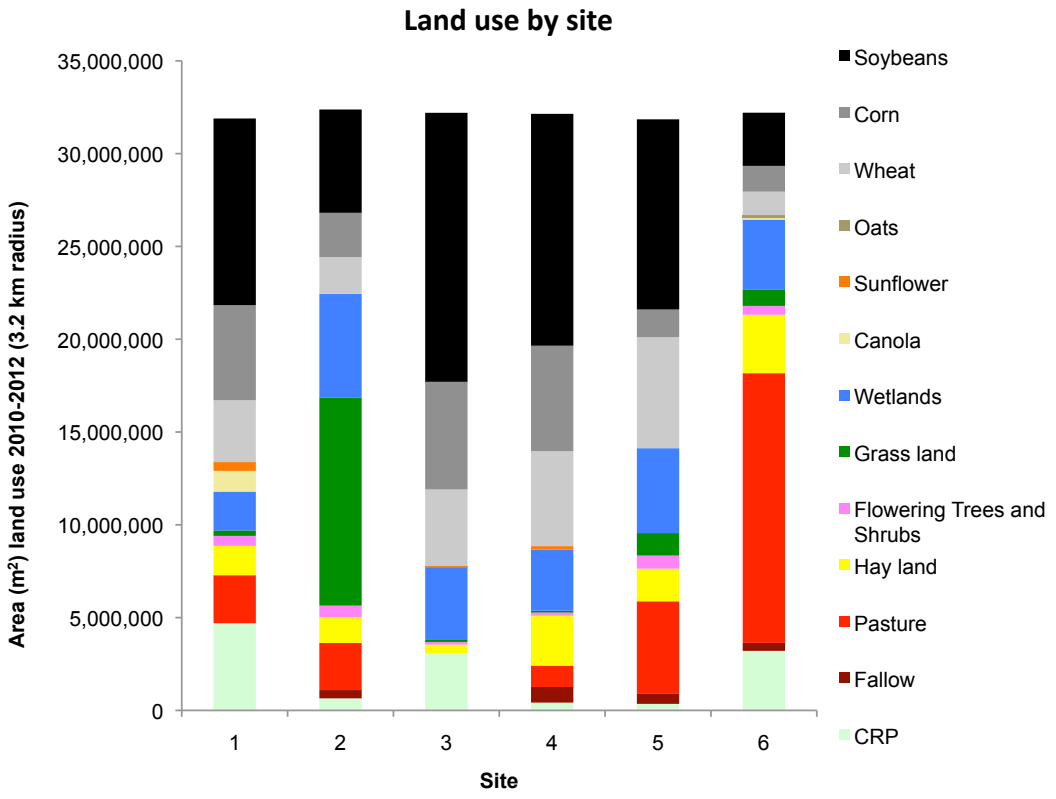

Land use by site
Area (m²) land use 2010-2012 (3.2 km radius)
35,000,000
30,000,000
25,000,000
20,000,000
15,000,000
10,000,000
5,000,000
0
1
2
3
4
5
6
Site
Soybeans
Corn
Wheat
Oats
Sunflower
Canola
Wetlands
Grass land
Flowering Trees and Shrubs
Hay land
Pasture
Fallow
CRP

Figure 2.3

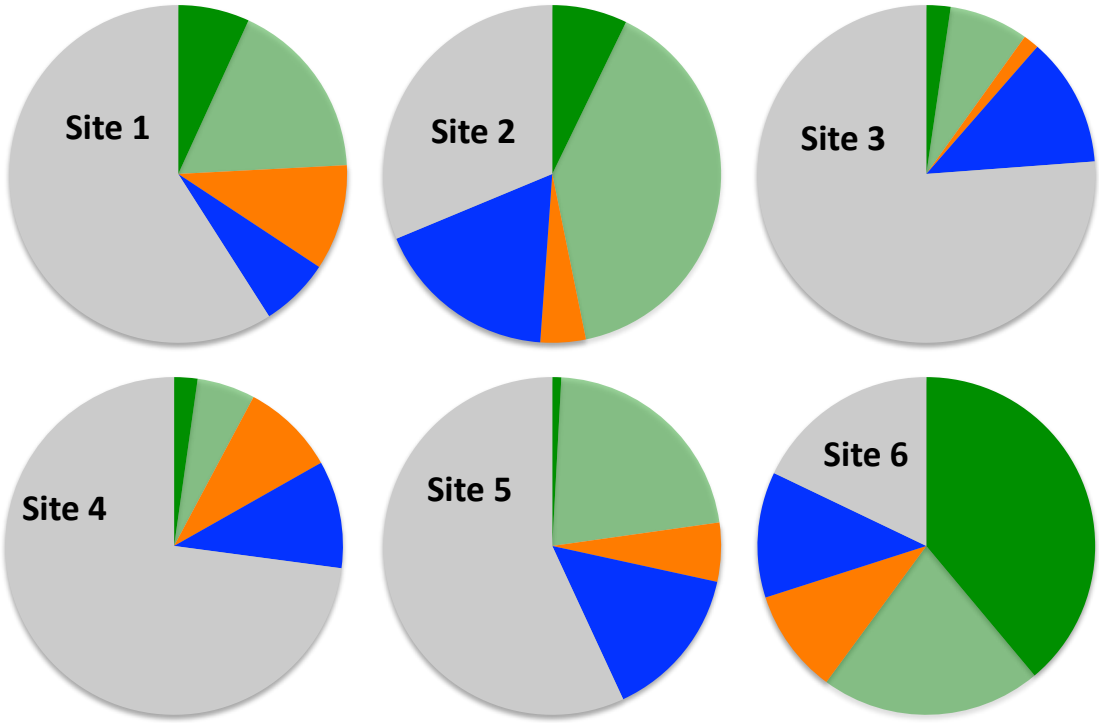
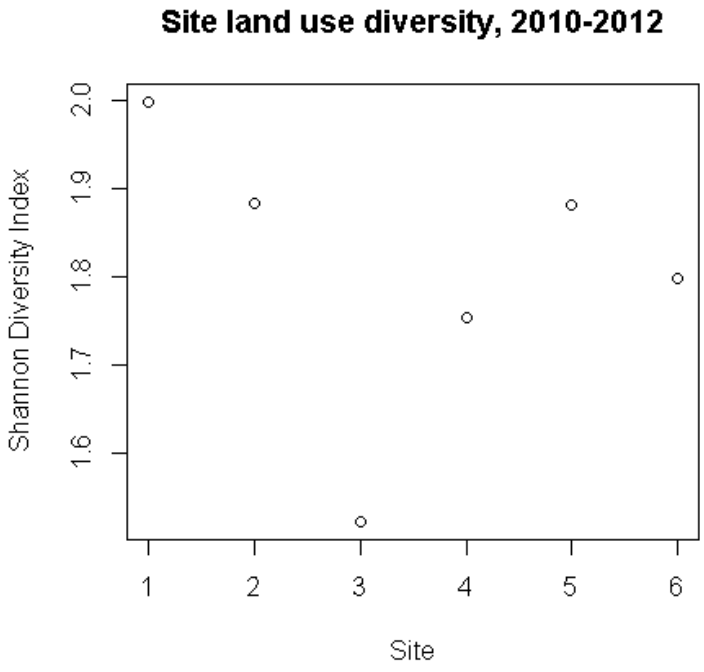


Figure 2.4


Site land use diversity, 2010-2012
Shannon Diversity Index
1.6
1.7
1.8
1.9
2.0
1
2
3
4
5
6
Site

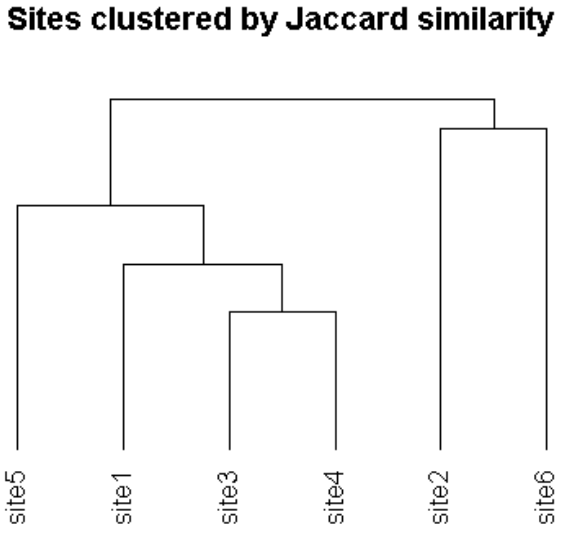
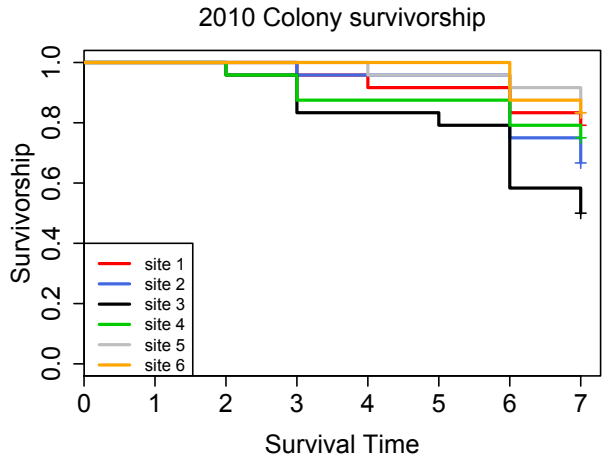
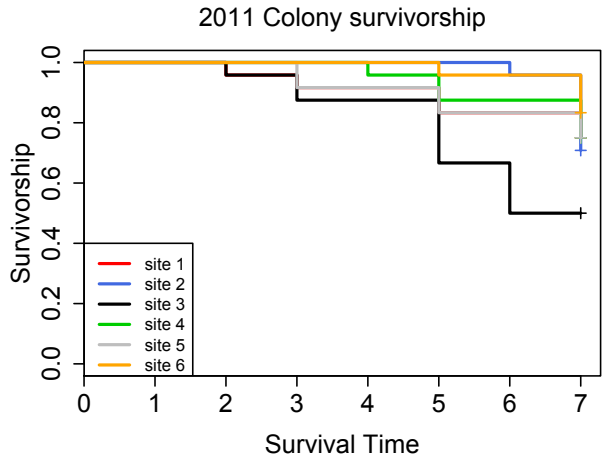
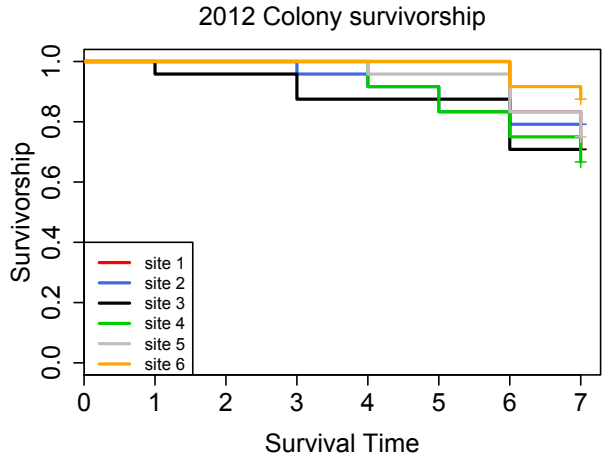

Sites clustered by Jaccard similarity
site5
site1
site3
site4
site2
site6

Figure 2.5


2010 Colony survivorship
Survivorship
0.0
0.2
0.4
0.6
0.8
1.0
0
1
2
3
4
5
6
7
Survival Time
site 1
site 2
site 3
site 4
site 5
site 6


2011 Colony survivorship
Survivorship
0.0
0.2
0.4
0.6
0.8
1.0
0
1
2
3
4
5
6
7
Survival Time
site 1
site 2
site 3
site 4
site 5
site 6


2012 Colony survivorship
Survivorship
0.0
0.2
0.4
0.6
0.8
1.0
0
1
2
3
4
5
6
7
Survival Time
site 1
site 2
site 3
site 4
site 5
site 6

Chapter 3

Colony-level analysis of the effects of land use on honey bee health and survival

Matthew Smart

3.1 Introduction

The managed European honey bee (*Apis mellifera*) is of critical importance for insuring the future availability and security of food in the United States and worldwide. In the U.S. alone, this single species of bee is responsible for the pollination of around 130 crops (McGregor, 1976), adding an estimated $15 billion annually to the value of honey bee-pollinated crops (Morse and Calderone, 2000). While recent declines in pollinator species have been documented across the globe, the phenomenon of sustained, severe annual losses of managed, commercial honey bee colonies (and the phenomenon of Colony Collapse Disorder, CCD) has mainly been confined to North America and parts of Europe (NRC, 2006; vanEngelsdorp et al., 2008; Potts et al., 2010). More specifically, annual losses for commercial beekeepers in the U.S. have hovered around 30% since 2006-07, with a low of 22.3% in 2011-12 and a high of 40.1% in 2012-13 (vanEnglesdorp et al., 2007; vanEnglesdorp et al., 2008; vanEnglesdorp et al., 2010; vanEnglesdorp et al., 2011; vanEnglesdorp et al., 2012; Spleen et al., 2013; Steinhauer et al., 2014).

Several factors have been implicated (e.g. Cox-Foster et al., 2007; vanEngelsdorp et al. 2009a; vanEngelsdorp et al., 2013) as potentially contributing to losses of honey bee colonies that may be broadly categorized into three groups acting alone or in conjunction with each other: 1) Poor diet brought on by inadequate and/or deficient forage resources (Naug, 2009b; Alaux et al., 2010a; DeGrandi-Hoffman et al., 2010;

Alaux et al. 2011; Di Pasquale et al. 2013), 2) exposure to environmental and in-hive pesticides (Johnson et al., 2010; Mullin et al., 2010; Boncristiani et al., 2012; Pettis et al., 2012; Wu et al., 2012; Johnson et al., 2013a; Johnson et al., 2013b), and 3) pests, pathogens, and microbes (Amdam et al., 2004a; Cox-Foster et al., 2007; Higes et al., 2008; Antunez et al., 2009; Alaux et al., 2010b; Zhang et al., 2010; Chaimanee et al., 2012; Martinson et al., 2012; Goblirsch et al., 2013). No single factor has been shown to occur in all cases of failing colonies, and therefore, a more complicated and nuanced picture of the dynamics occurring inside the hive has emerged – one in which many factors working together and/or at various times of the year, may lead to colony failure (Runckel et al., 2011; Pettis et al., 2012; Wu et al., 2012; DeGrandi-Hoffman et al., 2013; vanEngelsdorp et al., 2013).

Previously (in Chapter 2 of this dissertation) I showed that the area of potential honey bee forage, specifically the area of uncultivated, un-mowed bee forage within a 3.2-km radius of an apiary, had a significant impact on the survival of commercial honey bee colonies in the Prairie Pothole Region of North Dakota. Here, I tested the effects of the same six forage areas on various health measures of the honey bee colonies over a three-year period. The goal was to identify the most informative colony-level health measures, and time points for taking those measures, to infer and predict the proportion of colonies that survive within the different foraging landscapes.

3.2 Materials and methods

3.2.1 General Methods

A total of 144 colonies (24 in each of six sites) were extensively assessed and sampled over the course of 3 years (May 2010-Mar 2013) for a number of commonly used measures indicative of colony robustness and health (Delaplane et al., 2013a). Sampling and assessments occurred approximately every six weeks year round. Colonies were positioned in North Dakota apiaries surrounded by landscapes of varying land use patterns (Chapter 2) and, therefore, varying amount and quality of forage throughout the growing season (May-September). Colonies were moved to holding locations in California in October of each year before being moved into almond orchards for pollination beginning in February of each following year. Assessment/sampling locales, then, were in North Dakota during the months of May, July, August, and September, and in California holding yards (November and January of 2010-2013), and almond groves (March of each year).

A certain amount of overwinter colony mortality is reasonably expected to occur in any beekeeping operation. Thus, the cooperating beekeeper placed new replacement colonies into the position of dead colonies each year in May, returning the total number of assessed colonies at each site to 24 at the beginning of each season in North Dakota. In addition, every colony was re-queened annually in April-May by removing the old queen and introducing a newly-mated queen to the colony. Thus, in beekeeping terms, the colony year began with the introduction of the new queen (May sampling time) and ended after the colonies completed the almond pollination contracts (the following March).

Standard beekeeper practices were also implemented on every colony by the beekeeper to control of pests and diseases. *Varroa destructor* mite populations were suppressed by laying a shop towel soaked in a 2:1 canola oil:Tactic® (10% amitraz) solution over the top bars of the top box each spring and fall (May and August-September) for 14 days. *Nosema* spp. were controlled using Fumagilin-b® (Medivet Pharmaceuticals Ltd.) delivered in 1:1 sugar syrup twice per year in September and February. An antibiotic, Tylan® (Dadant and Sons), was used prophylactically to suppress bacterial infection, namely the causative agent of American Foulbrood, *Paenibacillus larvae*, and was delivered to the colony in sugar syrup twice per year with Fumagilin-b®. Finally, the beekeeper maintained a comb rotation scheme wherein one frame of foundation was inserted into, and one old frame removed from, each colony per year

3.2.2 Colony population size

The bee population was recorded for each colony by estimating the total number of frames (wax combs contained within a removable wooden frame) covered with bees within each colony. Bee population was the first measure taken after opening a colony on each inspection in the following manner. With the lid removed, the observer visually observed the combs in the top box from above and below, and the bottom box combs from above to approximate the number of combs completely covered by bees. This initial estimate was confirmed as the observer worked their way completely through the colony, comb by comb and was revised as necessary. If, for example, the tops of the combs within the bottom box appeared to be covered with bees but, as the combs were

removed and inspected, were depauperate of bees, the count of number of combs covered with bees was revised.

The amount of brood in the pupal stage (sealed with a wax capping) present in the colony was determined by placing a grid composed of 32 squares over each side of every frame within each colony on each sample date (similar to Delaplane et al. 2013a). The number of grid squares containing worker pupae (i.e. not drones) on each frame was then summed to determine the total number of squares in the entire colony. The number of squares was then converted to number of combs by dividing the total number of squares by 64 (to account for coverage of both sides of each comb).

3.2.3 Colony nutritional resources

All colonies were given additional boxes in which to store honey ("honey supers") by the beekeeper as needed over the course of the summer. The collaborating beekeeper maintained an adequate number of honey supers for colonies to continue storing honey. The amount of honey produced by each colony per year was determined by weight when the supers were removed in the fall.

Total pollen stored in each colony was approximated by overlaying a grid (as described for sealed brood estimation) on each frame, summing the number of grid cells containing pollen, and dividing by the total number of possible grid cells (64 per frame) (Delaplane et al. 2013a). This measurement was taken only in August and September of each year as an indicator of subsequent overwintering survival and spring success (Farrar, 1936; Jeffree, 1956; Jeffree and Allen, 1957; Schulz et al., 1998; Keller et al. 2005a, 2005b).

An additional measure of pollen collection was taken by maintaining three sentinel colonies at each of the six locations for pollen collection; these colonies were not included in the 24 regularly assessed colonies. The three colonies per site were fitted with pollen traps that, when opened, forced returning foraging honey bees to walk through the screens upon entering the hive (Delaplane et al., 2013b). The screens dislodged the pollen loads from the bee hind tibiae into a pollen collection drawer before the bee entered the colony. Traps were open for a 24-hour period 3-6 times per summer (six in 2010, five in 2011, three in 2012), and subsequently pollen was collected into a plastic bag and placed in a cooler on dry ice for shipping. Upon arrival at the lab at the USDA-ARS-Bee Research Lab in Beltsville, Maryland, samples were stored at -80C until analyzed. Total weight of fresh pollen (mass in grams) was determined in the lab for each sample date, colony, and site.

3.2.4 Floral pollen source identification

A separate 3-gram fresh pollen sample from the sentinel colonies at each site and for each date was sorted first by color to narrow down taxonomic diversity, then was subsequently identified using light microscopy for taxonomic plant of origin. Attempts were made to identify pollen to the lowest taxonomic level possible, though in many cases certain pollens could only be identified to genus or family, or were not able to be identified. Dividing the fresh pollen mass by the proportion of a given species (or genus or family) within a sample enabled us to provide an estimate of the amount of pollen collected from various taxa, and therefore the relative importance of those pollen species to bee health over the course of each summer foraging season.

3.2.5 Pesticide residue analysis of pollen samples

A subsample of the collected fresh pollen from the sentinel colonies on each date and site was sent to USDA-AMS-National Science Laboratory in Gastonia, NC for pesticide residue analysis. Results were reported in ppb on each date for 174 commonly used insecticides, fungicides, herbicides and metabolites. Pesticide residues in ppb were converted to a pollen hazard quotient (PHQ), defined as the average annual ppb of a given pesticide divided by its contact LD_{50} (few oral LD_{50} data exist for many of the pesticides detected in the study) (Stoner and Eitzer, 2013). Additionally, using contact LD_{50} values is more conservative because they are usually less toxic (higher LD_{50}) compared to oral LD_{50} values for the same pesticide. Contact LD_{50} values used for calculating PHQ were determined by averaging reported values from 3 studies (Mullin et al., 2010; Stoner and Eitzer, 2013; Sanchez-Bayo and Goka, 2014; and the EPA Office of Pesticide Programs Ecotoxicity Database USEPA, 2014). Importantly, pollen hazard quotients fail to consider synergistic or inhibitory interactions between and among pesticides. However the PHQs do allow for a comparison of the relative risk of pesticide exposure across sites in a much more biologically relevant manner compared to strictly comparing sums of ppb which does not take into account the variable toxicity of different chemicals.

3.2.6 Colony queen status

Queen status was determined for each colony during each assessment. Every effort to visually observe the queen's presence was made, however, queens often were

not seen, particularly during sample dates with large colony sizes and activity. In such cases, evidence of the queen (occurrence of eggs and young larvae) were noted and used as a proxy for the presence of a laying, normally functioning queen. Abnormalities in egg-laying patterns were also recorded when observed. Because queen status was recorded on each assessment date, it was possible to characterize the prior status and/or abnormalities that may have contributed to the loss of those colonies. For example, queen problems included colonies in which the queen had depleted her stored sperm and was laying only unfertilized drone eggs (drone layer), or the old queen had died and the colony either was not able to raise a new queen from existing larvae (queenless), or the workers had begun laying eggs (laying worker). In a few instances a new unmated queen was observed in the nest (virgin) but the colonies ultimately failed to survive.

3.2.7 Colony mortality

Colonies that died were grouped into four categories. The first three categories were colonies that laying queens but nonetheless died in the following ways: 1) spontaneously, where on the previous sample date a given colony appeared normal, robust, and queenright prior to its discovery as dead, 2) by dwindling, where colonies were observed to lag or regress in population size over time before ultimately dying, and 3) by disease, where obvious disease symptoms were observed previously. The latter category was only observed once, in which the colony in question presented with a heavy chalkbrood (fungal pathogen) infection on the previous assessment date. Category 4 contained colonies that had queen problems prior to death.

3.2.8 Parasites and pathogens

Adult bees were assayed for the occurrence and infestation/infection rate of commonly occurring parasite and pathogens. *Varroa* mite infestation rate per 100 bees was determined via alcohol wash to dislodge adult mites, after which the total number of mites and bees were counted. In the field, a single comb (thoroughly inspected to eliminate sampling of the queen) from the brood chamber was shaken into a plastic tub and approximately 300 adult bees were collected into bottles containing 70% ethanol. Once back at the lab, bottled bees and mites were mechanically shaken for 60 min to completely dislodge mites from adult bees. After the 60 min of shaking, mites and bees from each sample bottle were counted to arrive at a final *Varroa* infestation rate per 100 bees.

Nosema spp. spores per bee were determined from the same bees as *Varroa* mite infestation. One hundred bees were ground in 100 mL of water using a mortar and pestle. Ten microliters of this solution was pipetted into a hemacytometer and the number of spores per bee was determined using the standard procedure of counting cells/spores in a hemacytometer and estimating the total number in the sample (Human et al., 2013).

A separate sample of bees was collected from the brood chamber of each colony to detect the occurrence of viruses. The samples were placed immediately on dry ice in the field for shipping and stored in the lab at -80°C for later analysis. Total RNA was isolated from 50 bees using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. DNA was removed using DNAse I in an 11 µl reaction containing 8 µl (1.5 µg) total RNA, 10 U DNAse I (Invitrogen) in appropriate buffer, 20 U RNAseout (Invitrogen), poly dT(12-18), random heptamer oligonucleotides, and 2 mM

dNTP. The DNAse reaction was performed at 37ºC for 1 hr. followed by 75ºC for 10 min. Then, first-stand cDNA was synthesized by using 100U Superscript II Reverse Transcriptase (Invitrogen) and incubation at 42 ºC for 50 min followed by 15 min at 70 ºC. The cDNA was diluted 1:5 in nuclease free water (~100 ng/µl). Quantitative PCR was performed in a 20 µl reaction mixture consisting of 1X SsoAdvanced™ SYBR® Green supermix (Bio-Rad), 0.2 µM of each primer, and 1µl (~100 ng) of cDNA template. The oligonucleotide primers for qPCR are shown in Table 3.3. The reaction was carried out in 96-well plates using a Bio-Rad Icycler (Bio-Rad Crop., Hercules, CA.) programmed with the following temperature profile: 95ºC for 30sec followed by 50 cycles of 95ºC for 5 s, 60ºC for 30 s, melt curve from 65–95ºC in 0.5ºC/5s increments. The melt curve dissociation was analyzed to confirm each amplicon.

3.2.9 Colony supplemental feed

Colonies were nutritionally supplemented at certain times of year by the beekeeper with protein (to simulate pollen) and sugar syrup (to simulate nectar/honey). All colonies received Megabee® as their supplemental protein source twice in spring (a single 1lb. patty in April and May). Additionally, each autumn (August and September) half (12) of the colonies at each site were experimentally given additional protein supplementation in the form of Megabee® protein patties placed on the top bars of the bottom brood box for the bees to consume. This addition of a 1 lb protein supplement occurred twice, in August and September, each year and was included to determine whether feeding additional protein in the fall would lead to better outcomes for those colonies over the winter in terms of health and survival. Sugar syrup was provided to

colonies in spring and fall, with approximately 3.75 gallons given to each colony in each season.

3.2.10 Statistical Analysis

Repeated measures analyses of variance were conducted for each colony measure across all dates to determine at which time points (if any) a given predictor variable significantly differed from site to site. Significant differences in factors at the $\alpha \leq 0.05$ level were further examined using the conservative Tukey's multiple comparison test to determine on which dates variables actually differed. This ANOVA, in addition to investigator specialized knowledge coupled with biological relevance acted as starting points to inform selection of particular variables and sample dates to include in statistical modeling. Parameters deemed biologically relevant to the question of *which colony measures are useful in predicting colony overwintering survival* were included in statistical modeling using R version 3.1.1 (2014-07-10) and the lme4 package. A model was created with the response variable: arc-sine square root-transformed annual proportion of colonies surviving, and the factors site and year were included as random effects. All other colony level predictors were considered as fixed effects. The initial model contained all of the following factors: frames of bees in August and September, frames of brood in August and September, frames of pollen in the fall (average of August and September), average mass of fresh pollen collected per day, *Varroa* infestation in August and September, *Nosema* level in September, pollen hazard quotient, and the September levels of BQCV, DWV, and KBV. Backward selection from this large model was employed wherein after running each model, the least significant factor was removed

and then the model was re-run. This procedure was carried out until all remaining factors in the model were significant ($p \leq 0.05$). Akaike's Information Criteria (a standardized method for comparing alternate statistical models) was then used to select and confirm that the best of the available models was chosen.

Diversity of pollen (using the proportion of families and genera) was analyzed by calculating the Shannon-Weiner Diversity Index (H) for each site, defined as: $H= -\sum[(pi)*\ln(pi)]$, where pi in this case is the proportion of families or genera at a site relative to the total number of families or genera across all sites. A Jaccard similarity coefficient was also calculated to group and assess similarities between sites.

For the colony protein treatment, analysis of variance was conducted to determine the effects of fall protein supplementation on hive health measures as described above and the design of this study, then, was a split-plot, repeated measures experiment, with the whole plot factor being the landscape in which the colonies were placed, and the sub-plot factor being (+/-) fall protein supplementation.

3.3 Results

3.3.1 Statistically modeled measures correlated with colony survival

The large model, which included all measured variables, was pared down to include only biologically relevant and statistically significant measures. The three colony-level health measures that most predicted colony survival over the three years of the study were: 1) the average number of combs containing pupae (sealed brood) in September, 2) the average weight of fresh pollen collected per day over the summer, and 3) the average *Varroa* mite infestation level in September (Table 3.1). Increasing

amounts of pupae and pollen collected had positive influences on colony survival, while higher *Varroa* infestation levels in September were associated with decreased survival (Table 3.1). The response variable, annual proportion of colonies surviving by site over the three-year study, is shown in Table 2.5. Site six experienced the best survival in all three years of the study (along with site 5 in 2010), while site 3 experienced the poorest survival in 2010 and 2011, and second lowest in 2012.

3.3.2 Colony-level measures of health

The results of the ANOVA for each colony measure (frames of bees, frames of brood, frames of pollen and honey production, *Varroa* mite infestation, *Nosema* spp. spores per bee, and viral titers) on each sample date over the three-year study period are shown in Table A1.1. These data were primarily used to inform selection of factors to include in statistical modeling above. Graphically-depicted significance for each colony metric among all sites and sample dates are presented in Figures A2.1-A2.12.

3.3.2.1 Population of bees and brood

Colony adult population sizes, measured in combs of adult bees, peaked in July-September and declined over the winter until foraging was resumed in March of each year (Figure 3.1). Colony populations were smallest in January. These seasonal variations in colony population led to significant interactions between site and date ($F_{100,2383}=2.58$, $p<0.0000$), However, significant differences among sites within a date only occurred in July of 2010 between site 2 and sites 3-6 (Figure A2.1).

Maximum brood area, measured as combs containing pupae (sealed brood) occurred in July-August of each year (Figure 3.2), and the minimum occurred from November through January, at which times little brood rearing occurs in honey bee colonies. There were significant interactions between site and date ($F_{95,2561}$=1.82, p<0.0000) in brood areas for July of 2010 and August of 2011 (Table A1.1), meaning a given site did not have consistently larger colonies across all dates.

3.3.2.2 Colony nutritional stores

Honey production interacted by site and year ($F_{10,414}$=7.1, p=2.8x10^{-10}, Table A1.1). Sites 1 and 6 generally had the greatest honey yields, while sites 2 and 3 generally produced the least. By year and across sites, honey production was greatest in 2012 and least in 2011.

Site 6 had relatively more pollen stored in combs on the two late summer sample dates and years, though other sites also had large stores (e.g. site 3 in August 2010, sites 3, 4, and 5 in August 2011, sites 3, 4, and 5 in September 2011, and site 2 in September 2012). Site 2 had consistently low pollen stores, at least in the first two years (Figure 3.3). There was an interaction between site and date ($F_{25,801}$=3.15, p<0.0000) for pollen stores, meaning colonies at particular sites did not always bring in more pollen across all date (Table A1.1).

Pollen collection, the fresh weight of incoming pollen collected in traps per 24-hour sample period, was measured more frequently than area of pollen stores within the colony, and thus represents a real-time colony collection effort, rather than an end-of-summer culmination, as was the case for stored pollen. A significant interaction was

detected between site and date ($F_{65,168}$=1.42, p=0.038, Table A1.1). Due to the high degree of variance among the three colonies within a site on many sample dates (Figure 3.4a-c), only one significant site difference was found (between sites 1 and 5 on August 25, 2010). Nevertheless, the statistical model indicated that the weight of pollen collected by colonies across sites had a significant impact on annual survival.

3.3.2.3 Pollen identification

A total of 18 different plant families including 33 genera were detected in the forager-collected pollen pellets over the three years of the study (Table 3.2). Three families (Asteraceae, Brassicaceae, and Fabaceae) together made up the majority of bee-collected pollen in these landscapes, providing up to 57%, 26%, and 81%, respectively (39-94% overall) of the total pollen collected across all six sites and three years (Table 3.2).

Fabaceae and Brassicaceae pollen tended to be available in the late spring through mid-summer, while Asteraceae became more predominant mid-summer through late summer. One genus of Fabaceae, *Melilotus* spp. (encompassing white and yellow sweet clover) was particularly persistent, in terms of bloom phenology (pollen present in samples from late June through early September), and dominant in proportion of the total pollen collected by the bees (Table 3.2, Site 1: 7-47%, Site 2: 13-66%, Site 3: 18-35%, Site 4: 9-45%, Site 5: 2-29%, Site 6: 2-39%), particularly in 2010 and 2011 (Table 3.2, Figure 3.4a-c).

Pollen diversity by site, date, and year in relation to the average amount of pollen collected per site and date and overall land use available in forage are depicted in Figure

3.4a-c. Across most sites and years, much of the diversity in pollen was contributed via a relatively small proportion of pollen (i.e. most of the pollen collected belonged to just a few families and genera). For example, in 2010 *Melilotus* spp. (Fabaceae), and *Sonchus* spp. and *Grindelia squarrosa* (Asteraceae) were particularly dominant. Dark green and orange in the pie charts depicted in Figure 2.3 indicate the proportion of land within the 3.2-km radius of each apiary that contained flowers, and represents a relatively small area around each site in food resources. Lands around site 6 contained the most flowers, and, at least in 2010 an 2011, bees collected more pollen at that site, while colonies collected relatively less pollen at sites 2, 3, and 4 in 2010, sites 2 and 4 in 2011, and sites 4 and 5 in 2012.

Cultivated plants including alfalfa, green bean, canola, sunflower, and soybean made up relatively little of the total pollen brought into colonies across sites (site 1: 8%, site 2: 12%, site 3: 3%, site 4: 8%, site 5: 10%, site 6: 17%). *Glycine max* (soybean) pollen specifically, though detected, was relatively rare, occurring only at site 2 (0.4% of all site 2 pollen in 2010), and site 3 (2% of all site 3 pollen in 2010) only. No corn pollen was detected. Many of the most commonly collected genera/species of plants identified through the pollen in this study were non-native to the United States, including *Melilotus* spp., *Medicago sativa* (cultivated), *Circium* spp., *Sonchus* spp., *Taraxacum officinale*, *Tragopogon* spp., *Centaurea maculosa*, *Cichorium* spp., and *Silene latifolia*. Several native species, and others that are potentially native depending on the species within the genera identified, were also found including, *Solidago* spp., *Grindelia squarrosa*, *Helianthus* spp. (cultivated or wild), *Vicia* spp., *Lathyrus* spp., *Trifolium* spp., *Phaseolus* spp. (cultivated), and *Lupinus* spp. (Table 3.2).

Pollen diversity analysis (Figure 3.5) indicated that the most diverse site in terms of trapped, forager-collected pollen was site 3 followed by sites 4, 5, 6, 1, and 2 at the family level (Figure 3.5a) and site 6 at the genus level followed by sites 3, 4, 5, 1, and 2 (Figure 3.5c). Interestingly, two of the most diverse sites here, sites 3 and 4, were found to be the least diverse in terms of land use (Figure 2.4). Analyses at both the family and genus levels indicated that site 2 was the least diverse, despite possessing relatively high diversity in terms of land use (Figure 2.4).

Jaccard similarity analysis indicated that at the pollen family level, sites 1 and 6 formed a distinct cluster, with sites 3, 4, and 5 diversity occurring intermediately (Figure 3.5b). Site 2 diversity was very low and fell into an outgroup, most dissimilar to any of the other five sites. Jaccard similarity at the genus level produced three distinct clusters between two sites each, including sites 1 and 2, and sites 4 and 6, and sites 3 and 5 (Figure 3.5d).

3.3.2.4 Pesticides

Pesticide residues from agricultural and beekeeper applications were detected in the fresh pollen collected from the 18 sentinel colonies (three per site) throughout the season across all sites and years (Table 3.4). Although colonies were exposed to a number of pesticides over the three years, there was no significant impact of pesticide exposure through pollen on colony survivorship in this study.

A varying number of pollen samples were collected each year (six in 2010, five in 2011, and three in 2012). Thus the pesticide residue in parts per billion (ppb), used to calculate the pollen hazard quotient (PHQ) of each chemical detected at each site was

averaged across the three years of the study, except when a chemical was detected only one time (Table 3.4).

3.3.2.4.1 Agricultural pesticides

Notably, no neonicotinyl insecticides were detected in any pollen samples over the three years even though the apiaries were located close to corn and soybean fields. Nine insecticides were detected that have high toxicity to bees, two organophosphates (OPs) and six pyrethroids (Table 3.4, red highlighted boxes). Of the two OPs, chlorpyrifos was the most commonly found, detected in pollen from sites 3 and 4 in 2010, and from all sites in 2011 and 2012. Methyl parathion, the other OP, was detected only at sites 3 and 5 in 2010. Of the seven pyrethroids detected (six of which have high toxicity to honey bees), cyhalothrin was the most commonly found, detected in pollen from site 4 in 2010, all sites in 2011, and site 2 in 2012. Bifenthrin, cyfluthrin, cypermethrin, and esfenvalerate were found sporatically across the sites and years. Deltamethrin was detected on only one sample date from site 6 in 2010. Chlorthanlonil, the seventh pyrethroid detected, has low toxicity to honey bees, and was detected five times (once at each of sites 1, 2, 4 and 6 in 2010, and once at site 1 in 2011). Chlorfenapyr (a partial systemic insecticide with high honey bee toxicity) was detected on only one sample date from site 6 in 2010, while endosulfan II and endosulfan sulfate (cyclodiene insecticides with moderate toxicity to honey bees) were each detected only once at site 4 in 2010. Finally, fenpyroximate, a pyrazole insecticide with moderate toxicity to honey bees, was found in pollen from all sites except site 3 in 2010, but was only detected at sites 1 and 5 in 2011 and 2012, respectively.

In addition to the aforementioned insecticides found in the study, agriculturally-applied fungicides and herbicides were detected in returning forager pollen loads. Overall, five fungicides (all with low honey bee toxicity) were detected but the most commonly found fungicide was carbendazim, detected at all six sites in June-August 2010, but only at sites 5 and 6 in 2011, and sites 1 and 2 in 2012 (detected on a single date at those sites in 2011 and 2012). The other four fungicides, pyraclostrobin (site 4 in 2010), tebuconazole (site 2 in 2011), trifloxystrobin (site 4 in 2012), and vinclozolin (site 6 in 2010) were each only detected on one sample date and site each. Finally, four sparse detections of three herbicides were found: oxyfluorfen (site 6 in August of 2010), pendimethalin (site 1 in June of 2010), and trifluralin (sites 3 and 4 in August of 2012 and 2010, respectively).

3.3.2.4.2 Beekeeper-utilized pesticides

Residues of six beekeeper-utilized pesticides were found in fresh pollen samples across all sites and years including the miticides/miticide metabolites: coumaphos, coumaphos oxon, fluvalinate, thymol, and 2,4 Dimethylphenyl formamide (DMPF) a breakdown product of the miticide, Amitraz. Paradichlorobenzene, a chemical used as a fumigant to deter stored beekeeping equipment pests, such as wax moths, was commonly detected at all sites, but only in 2011. The fact that it was only detected in one year suggests that the product may have inadvertently remained in honey supers that were added to colonies that year. The toxicities of thymol, DMPF, and paradichlorobenzene are not known. The other products have low or moderate toxicity to bees when applied individually, although synergies may occur when combined.

3.3.2.5 Queens

As new queens were introduced into each colony in the spring of each year, the life span of a colony was considered to extend from May of one year (when colonies were in North Dakota) to March of the following year (when colonies were in almond orchards in California). Figure 3.6 shows the status of queens across sites and years in colonies immediately prior to being reported as dead. Most colonies (over 60%) that died had a laying, functional queen on the previous inspection. However, 31-42% of the colonies that died had apparent queen problems prior to mortality. As reported in the Chapter 2 survival analysis, site 3 experienced the lowest survival in 2010 and 2011, while site 6 experienced the greatest survival in all years. These differences in survival were related to the overall availability of bee forage around those sites (Chapter 2).

3.3.2.6 Colony mortality

Most colonies (over 60%) that died had a laying, functional queen on the previous inspection. The cause of death of the majority of colonies was categorized as dying spontaneously, dwindling over time, or from disease (Figure 3.6). Only one colony died of an obvious disease, *Ascophera apis* the causative agent of chalkbrood, in 2010.

3.3.2.7 Parasites and pathogens

Varroa mites were controlled by the beekeepers using Amitraz. Infestation rate (mean no. mites per 100 adult bees) steadily increased over the course of each summer

after treatment in May of each spring (Figure 3.7, Figure A2.4). In 2010 and 2011, mite levels never exceeded 1 mite/100 bees, while in 2012, mite levels were slightly higher. Importantly, mite infestation apiary averages, and infestation rates in most colonies within apiaries, were suppressed to levels well below those known to cause harm to colonies (e.g. > 5 mites/ 100 bees). Repeated measures ANOVA revealed a significant interaction between site and date ($F_{85,2375}$=3.8, p<0.0000), indicating that colonies in one site did not consistently have the lowest or highest mite levels over time. The differences in *Varroa* levels across sites only occurred in August-January of the third year (Figure A2.4). Peak infestations were reached by August at most of the sites (with the exception of site 1 where peak mean levels were reached in September). Treatments were applied again in September, which, coupled with the natural decline in colony brood-rearing occurring in the fall, led to decreased *Varroa* infestation levels through January. By March, *Varroa* levels were once again increasing before treatments were applied in May of each year.

Treatment of *Nosema* spp. with Fumagilin-b® occurred in February and September of each year. Results of repeated measures ANOVA show a significant interaction between site and date ($F_{85,2374}$=3.5, p<0.0000), occurring in May 2010, July, August, and November of 2011, and July of 2012 (Figure A2.5). *Nosema* spp. infections typically peak in colonies over the late winter-spring and decline naturally over the summer, regardless of treatment. We observed peak levels across all sites in May-August and minimum levels in November-March, likely aided by treatments in September and February (Figure 3.8).

Viral titers varied greatly over the course of the study, both across sites and years (Table A1.1, Figures A2.6-12). Certain viruses including ABPV, CBPV, and KBV occurred at low levels throughout the year, while BQCV was present at relatively high levels year round. Still others, e.g. DWV, showed a strong seasonal distribution, likely aided by *Varroa* mite transmission, and reached peak mean levels in September of each year. Finally, IAPV and SBV both peaked in July of each year before titers declined naturally over the summer. Significant interaction between site and date occurred for most of the viruses measured, including ABPV ($F_{65,410}$=2.85, p<0.0000), BQCV ($F_{65,410}$=1.95, p<0.0000), DWV ($F_{65,410}$=1.88, p=0.0001), IAPV ($F_{65,410}$=2.12, p<0.0000), and SBV ($F_{65,410}$=1.47, p=0.014) (Figure A2.6-A2.12). For CBPV and KBV, the only significant effect was sample date (CBPV: $F_{13,475}$=7.62, p<0.0000; KBV: $F_{13,475}$=19.04, p<0.0000), meaning sites and dates varied in the presence and prevalence of viruses.

3.3.2.8 Supplemental protein feeding

We considered the potential longitudinal effects of August and September protein supplementation to extend only through the January sampling date of the following year. By the next March sampling date, colonies were able to forage for fresh environmental pollen in almond orchards. Supplementing colonies with commercial protein diet in the fall did not differentially affect colony survival. Protein feeding in the fall was, however, correlated with higher *Varroa* mite infestation levels by the next January (site by date interaction: $F_{17,2447}$=1.66, p=0.044) but only in 2012 (higher *Varroa* levels in supplemented vs. non-supplemented colonies, p=0.05).

3.4 Discussion

This study identified significant measures of the health of honey bee colonies located in North Dakota during the summer months that predicted colony survivorship of the colonies after they were moved to California to pollinate almonds. Out of 13 measures that were entered into the initial model, one three were significant in predicting future colony losses across the six sites: 1) the combs of sealed brood (pupae) in September, 2) average amount of fresh collected per day over the summer, and 3) *Varroa* mite infestation levels in September (Table 3.1).

The remaining colony measures, including those related to colony size, parasite and disease occurrence and levels, and pesticide exposure among sites, failed to impact survival in a statistically significant manner (Figures A2.1-12). One potential reason for this finding may be because the beekeeper regularly and effectively controlled diseases and parasites, and pesticide exposure may not have reached the levels of acute exposure.

3.4.1 Population of bees and brood

The amount of developing brood, measured as the area of pupae (sealed brood) the colonies contained in September, irrespective of site, was predictive of colony survivorship in the following spring. Colonies with larger brood areas in September tended to survive longer. A large brood area in fall leads to more long-lived winter bees (Mattila and Otis, 2007), and, particularly when mite infestations are low and honey stores are abundant, this larger colony size can lead to more successful survivorship over the winter due a larger population contributing to better thermoregulation of the overwintering cluster.

Adult bee population was not predictive of colony survivorship, but this measure may have been confounded by the time of day when the assessment was made, and whether the ambient temperatures caused bees to cluster within the hive boxes. For example, more bees generally can be found in a colony in the early morning compared to the late morning or afternoon when more bees are involved with foraging in the field. On the other hand, bees may have been clustered and less mobile in the early morning, making it appear as if fewer combs were actually covered by bees. Brood area, on the other hand, was measured using a grid to estimate total area pupating brood, which was very obvious when inspecting combs. There were only a few significant differences in colony brood areas among sites on any given date (July 2010: site 1 vs. 6 and site 2 vs. 3, 6; August 2011: site 3 vs. 6), indicating that colonies grew at similar rates across all sites and years during the summer months.

3.4.2 Colony nutritional stores

Colonies, in general, maintain modest yet consistent stores (around 1 kg) of pollen in the hive throughout the growing season, enough to last for approximately 1 week in the event of a pollen dearth scenario (Seeley, 1995). The demand for pollen in the hive remains relatively stable as the population grows and then declines over the summer. At the same time, considerable fluctuation exists in the environmental availability of pollen due to plant phenology and weather, hence the stable ~1 kg of stored pollen (Seeley, 1995). We observed some differences in colony pollen storage by site (Figure 3.3, Figure A2.3) but the differences that did occur were only detected in August (i.e. none in September) of each year 2010-2012. Colonies presumably did not collect and store

excessive amounts of pollen in the hive due to the need for balance between food (nectar and pollen) storage and brood production. Seeley (1995) suggested an equation relating a colony's pollen production to three variables:

$$C = \frac{NL}{T}$$

where N is the number of foragers collecting pollen, L is the mean pollen load size, and T is the average foraging trip time. Each of these variables may be adjusted by colonies to maintain the appropriate amount of pollen in the hive (Seeley, 1995). Studies also suggest that foraging honey bees do not assess pollen quality or preferentially collect pollen of higher protein content (Pernal and Currie, 2001; Pernal and Currie, 2002). However, pollen collection is heritable, thus increased pollen collection may be selected for genetically (Page and Fondrk, 1995; Waddington et al., 1998).

The land use surrounding each site varied, resulting in different amounts of floral resources that were available to the colonies (Tables 2.1 and 2.2). For example, the amount of land in CRP surrounding sites 3 and 6 was similar ($3070x10^3$ m^2 and $3209x10^3$ m^2, respectively) but the actual realized floral cover within the CRP lands was vastly different (24% at site 3 compared to 74% at site 6). This ground survey data coupled with the weight per day of collected pollen data (Figure 3.4) indicate that the land use surrounding certain sites, for example site 6, was much richer in flower abundance which led to greater total pollen availability but not necessarily pollen diversity, though much variation occurred within and among sites.

Greater floral abundance within a 3.2-km radius of an apiary likely made it easier for bees from, for example site 6, to locate and exploit pollen resources (Table 2.2). Honey bees from site 3 may have had to travel farther and/or locate and exploit smaller

patches of flowers, thus depleting greater stored colony resources in an effort to support their colony population growth requirements over the growing season. Thus, while colony populations may not have differed significantly between sites on most of the sample dates during the summer, end of season honey production and ultimately survival were significantly impacted as a result of the varying spatial patterns of resources availability between sites.

Honey production, previously discussed in Chapter 2, was correlated with the area of uncultivated potential bee forage in the surrounding landscape, and for example, the site with the greatest amount of this forage, site 6, also produced the most honey. Honey production varied across sites, but colonies in the fall across all sites were essentially "equalized" going into winter in terms of the total amount of honey stored in the hive because all excess (in boxes above the two brood chamber boxes) was harvested by the beekeeper. Therefore differences in overwinter survival were not likely to be due to factors such as honey production, or pollen storage (not significantly different in September).

3.4.3 Pollen identification

Members of the plant families Asteraceae and Fabaceae comprised the most commonly detected pollens in our study. No genera/species were detected at all sites in all years, emphasizing the challenge that the bees face in relation to the ever-changing availability of pollen resources in these environments. One genus, *Melilotus* spp., was present in all years and sites save one (site 4 in 2012), highlighting the relative importance and preference for this copious nectar- and pollen-producing "volunteer"

biennial plant. Further, colonies fed *Melilotus* spp. pollen have been previously shown to produce the most brood compared to several other single source and blends of pollen, and sweet clover was most preferred (highest consumption) by the bees (Campana and Moeller, 1977). Aside from *Melilotus* spp., most of the other plants from which pollen was collected by bees were also "volunteers" in the sense that they were not actively cultivated, but rather, were allowed to grow in pastures, hayland, grasslands, ditches, and CRP lands. In fact, cultivated plants (sunflower, alfalfa, canola, bean (*Phaseolus* spp.), soybean) comprised on average only 10% of the total pollen collected by bees across all sites and years. These small percentages put into perspective the heavy reliance of honey bee colonies on weedy flowering resources in these agricultural lands that are in no way actively managed for pollinators, and as a result, are susceptible to loss through herbicide use and mowing and degradation over time.

The data regarding diversity of pollen is somewhat anomalous on the surface. The most diverse sites in terms of land use (sites 1, 2, and 6) had the least diverse pollen at the family level. On the contrary, sites 3 and 4 (least diverse land use) were the most diverse for families and in the top three for genera of pollen (Figure 3.5). Interestingly, site 6 had one of the lowest family diversities but the highest genera diversity, and importantly had the best colony survival, honey production, and measures of nutritional and immune physiology (Chapter 4). The next most diverse sites (at the family *and* genus levels) were sites 3 and 4, which possessed the least diverse land use, most cropland, and lowest survival and honey production (Chapter 2).

Results of the pollen diversity analysis should be approached with caution (particularly at the genus level) as in some cases large proportions of pollen by family

were not further identified. For example, 42% of the pollen at site 2 in 2010 and 50% of the pollen at site 4 in 2012 were "other Asteraceae" (Table 3.2). Further, there was always a proportion of pollen not definitively placed in any family. For example 22% of the pollen from site 5 in 2012 was designated as "undetermined" in Table 3.2, which obviously had an impact on the diversity of pollen at that site and year.

The lack of correspondence between pollen diversity collected by bees and land use diversity available to them may be due to colony optimization in the use of patches of existing "known" resources and exploration, discovery, and recruitment for new resources. For example, ground surveys of flowers (Table 2.2) indicated that for many of the land use categories in which flowers were seen, sites 3 and 4 had some of the smallest areas of total flower cover (e.g. site 3: grassland, hayland, pasture; site 4: CRP, hayland, grassland). Further, sites 3 and 4 had some of the most flowers in ditches (a landscape feature that is widely distributed and ephemeral due to mowing regimes). When patches of flowers in the available landscape are small or widely distributed, honey bee colonies from a common apiary are able to exploit fewer resources from them, and are required to expend greater time and energy locating those patches. As small or widely distributed patches become exhausted of resources, foragers must locate alternative, and potentially even smaller, or sparser, patches in the landscape, or fly farther to find them. As a result, colonies from those sites may be brought into contact with a greater diversity of pollen and nectar than colonies that are able to forage on large patches of one or a few species of plant located closer to the colony.

Conversely, many of the flowers from sites 1 and 2 were detected in CRP, grassland, and hayland, where patch sizes were large and attractive, though potentially

composed of relatively fewer species. Large patches of flowers at site 6 occurred in CRP, hayland, and pasture and, further, site 6 also had relatively large areas of flowers in ditches and grassland. Quality land use *and* flower cover seems to have intersected at site 6, and may have enabled bees at that site to exploit those large patches of dense flowers (e.g. *Melilotus* spp*., Medicago sativa*) when present, and then move to available smaller patches composed of more diverse species later.

3.4.4 Pesticides

3.4.4.1 Agricultural pesticides

Exposure of foraging bees to contaminated pollen was relatively ubiquitous across the study apiaries, including at site two located in the Arrowwood National Wildlife Refuge. Several of the most highly toxic pesticides detected across all sites are prescribed for use on soybeans against soybean aphids (*Aphis glycines* Matsumura), including chlorpyrifos, cyhalothrin, bifenthrin, and esfenvalerate (Table 3.4). Casual observation of soybeans during bloom indicated that honey bees did not visit soybean flowers, although we did identify a small amount of soybean pollen from two sites in 2010 and, further, honey bees can and do visit soybeans in other states (e.g. Erickson, 1975; Gill and O'Neal, in review). Given the high prevalence of these chemicals in our pollen samples, particularly chlorpyrifos, foragers probably came into contact with pesticides via drift onto flowers near agricultural fields during or after application. Corn planting typically occurs between early May and early June, overlapping with the presence of honey bee colonies on the landscape. The dust produced during corn planting may contain high levels of the neonicotinoid pesticide clothianidin, and wind

can blow this toxic dust onto nearby non-target flowers or be taken up by roots of flowering plants that bees visit near fields (Krupke et al., 2012). In this study, we did not detect any neonicotinoid insecticides, including clothianidin, nor did we detect corn in the bee-collected pollen.

Given the low survival of colonies (Table 2.5) and high proportion of non-bee forage, including soybeans, at site 3 (Figure 2.1) the greatest pesticide exposure was expected to occur there. This was not the case as a greater total number of pesticides and a greater total PHQ was detected at site 6. However the PHQ at site 6 was inflated by the single detection of very high levels of deltamethrin (a pyrethroid) in 2010 (Table 3.4). Removal of deltamethrin from the PHQ calculation and re-running the model still resulted in pesticides (PHQ) not contributing significantly to survival, and site 3 remained as the site with the lowest PHQ. The most prevalent chemical detected at sites 3 and 4, the two sites composed of the greatest proportion of non-bee forage, was chlorpyrifos, which was detected in 71.4% and 57.1% of forager pollen load samples over the three years, respectively. These detections can be contrasted with the other four sites at which the most commonly detected chemical was thymol, a beekeeper-applied volatile acaricide, while chlorpyrifos was found in 21.4-57.1% of samples.

Overall, no clear relationships were observed between pesticide exposure and colony health and survival in our study, but as has become clear in recent years, the most insidious effects of pesticides occur at the sub-lethal level and involve effects on memory and cognition, colony development, decreased longevity, and interactions between pesticides and other colony stressors (Yang et al., 2008; Aliouane et al., 2009; Wu et al. 2011; Pettis et al. 2012; Wu et al. 2012; Feltham et al., 2014).

Contact LD_{50}, rather than the arguably more biologically appropriate measure, oral LD_{50} (because the pollen is ultimately eaten), was used because data on oral toxicity to honey bees is available for only a few of the pesticides detected. As a result, care should be taken in drawing definitive conclusions regarding deleterious effects of pesticides from our data, as the most likely route of exposure to contaminated pollen is orally rather than by contact. At the same time, however, contact LD_{50} values are generally higher (lower toxicity) compared to oral values, and therefore represent a more conservative estimate of the overall toxicity of the pesticide residues detected.

3.4.4.2 Beekeeper-utilized pesticides

Beekeeper-applied chemicals were some of the most prevalent chemicals detected in the trapped pollen. This is somewhat surprising considering several of the chemicals (e.g. coumaphos, fluvalinate) have not been used by the beekeeper for over 5 years, and the beekeeper had a regular comb-replacement regime. The finding of miticides in pollen may be explained by the fact that several of the compounds, and their breakdown products, used in the past by beekeepers are lipophilic and tend to remain in wax comb for indefinite amounts of time (Wu et al., 2011). As bees develop to maturity in the comb and subsequently interact as immatures and adults with nestmates, they come in contact with these compounds and transfer them around the hive on their bodies. As a result, the detection of many in-hive miticides in forager pollen loads is likely due to these residues being present on the cuticles of most of the bees in the hives. As foragers, the bees gather pollen loads onto their corbiculae at flowers, transferring the compounds from their cuticle to the pollen. This type of chronic exposure to pesticide residues can have myriad

detrimental effects on bees (Haarmann et al., 2002; Pettis et al., 2004; Burley et al., 2008), and, further, has resulted in resistant populations of *Varroa* mites to many of the limited number of miticides in the beekeeper toolkit (Elzen et al., 1998; Pettis, 2004).

3.4.5 Queens

Queen problems were a common issue across sites, despite the beekeeper replacing the queens in all colonies annually. Queenless colonies and drone layers were common (14-100% across sites and years, Figure 3.6) immediately before colony death, however it should be noted that queen-right colonies also experienced death, particularly at sites 1 and 2 in 2010, site 3 in 2011, and at all sites in 2012 (Figure 3.6). Depending on when queen loss (in the case of queenlessness) or drone laying queens occurrs, the beekeeper can salvage otherwise doomed colonies via queen replacement. Queen failure may occur for a variety of reasons including poor queen mating, low drone sperm quality, queen and/or drone pesticide and disease exposure, inappropriate beekeeper-mediated queen introduction, natural queen senescence, and other unknown factors involving a lack of colony acceptance of introduced queens (Tarpy et al., 2000; Haarmann et al., 2002; Tarpy and Seeley, 2006; Richard et al., 2007; Delaney et al., 2010; Tarpy et al., 2011; Tarpy et al., 2012; Collins and Pettis, 2013), and causes of queen failure are an active area of current honey bee research.

3.4.6 Parasites and pathogens

Parasites and pathogens are known to cause harm to colonies, and have been previously linked to colony losses and colony collapse disorder (CCD) (e.g. Amdam et

al., 2004a; Cox-Foster et al., 2007; vanEngelsdorp et al., 2013). We failed to detect any meaningful differences in the occurrence or levels of several common parasites and diseases in relation to differential land use. It may be the case that differential land use does not play a large role in affecting parasites and diseases in general. In a migratory beekeeping operation there are multiple times each year when all colonies are essentially "homogenized" and potentially afforded opportunities to transmit diseases and parasites among each other (e.g. during transport on trucks, in holding yards, in almonds). Additionally, in this study there might not have been a great enough degree of difference in land use surrounding the apiaries to detect parasite and pathogen differences, or interactions between pesticides and diseases (e.g. Pettis et al., 2012; Wu et al., 2012), as seen in other studies.

The beekeeper maintained an effective management strategy to control the most damaging parasite, *V. destructor*, as well as *Nosema* spp. and bacterial agents. Since many of the known honey bee viruses are associated with *Varroa* mites (that were effectively controlled), and also seem to occur seasonally and/or ubiquitously (Runckel et al., 2011) in the bees/hive without causing obvious deleterious effects, it is perhaps not surprising that no large differences among the sites were observed. On the other hand, it is known that nutrition interacts to some degree with the immune system (e.g. DeGrandi-Hoffman et al., 2010), thus poor nutrition could potentially result in bees/colonies exhibiting an increased susceptibility to certain diseases.

3.4.7 Protein Supplementation

Fall protein supplementation positively affected average September and January brood areas, however, this boost in brood production may have had the corresponding negative effect of providing a safe harbor for *Varroa* populations longer into the winter, as we saw significantly greater *Varroa* infestation rates in supplemented colonies in January of 2012. However, the levels of *Varroa* were still relatively low, and the survival of the supplemented colonies to March was not different from non-supplemented colonies. Based on this finding, prophylactic supplementation of fall colonies with protein is not recommended for in northern climates, due to the lack of any measureable impact on colony size, food stores, of improved survival, and the potential risk of increasing *Varroa* mite populations over the winter.

3.5 Conclusions

This study has shown that three colony-level measures were significant in predicting the survival of migratory honey bee colonies transported from North Dakota to California for almond pollination: 1) the average amount (g) fresh pollen collected over the summer, 2) the amount of sealed brood (pupae) in September, and 3) the *Varroa* mite infestation levels in September. These results stress the importance of bees' access to abundant forage in the surrounding environment, both for honey production and pollen collection, which drives and sustains the population growth of colonies. Most of the forage in this study occurred in uncultivated areas of the landscape including pasture, CRP, grassland, hayland, and ditches where weedy flowers predominated. Thus greater consideration should and could be made toward policies of shared use and management

reflecting the unique requirements of the beekeeping industry in these types of lands and region of the country.

In a practical sense, beekeepers should closely monitor and control mites by early September. Mite treatments the previous May will impact the number of mites that need to be controlled by September and beekeepers may need to learn to better control mites via better monitoring and more frequent treatments, though this can be logistically difficult due to the risk of using, and labeling laws forbidding the use of, many chemicals during honey production. This study highlights the difficulty for beekeepers in assessing whether colonies are healthy and will survive from easily obtained colony information gathered from routine colony inspections (e.g. colony size, colony stored pollen, honey production). In the next chapter, more targeted diagnostics, including measures of blood storage proteins, brood food gland size, fat body lipids, and immune system activation were used to more accurately assess colony health and to predict colony survivorship. The future of beekeeping may involve the use of more sophisticated laboratory measures to help assess colony health.

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3.7 Table legends

Table 3.1 Statistical model of colony level data. The response variable (proportion of colonies surviving) was arc-sine – square root transformed and the factors pollen mass, combs of pupae (sealed worker brood) in September, and *Varroa* mite infestation level in September were significant in the final model. Site and year were partitioned as random effects.

Table 3.2 Pollen identification and proportion of families/genera/species of detected pollens by year and site. A 3-gram sample of mixed pollen from each sample date was sorted by color and families/genera/species within each color were proportionally quantified and summed across all samples dates within each year.

Table 3.3 Status of colonies, and queen events, prior to mortality. Spontaneously dead colonies were those that "collapsed", i.e. appeared healthy and robust on the sample date prior to being found dead. Dwindling colonies decreased in population size gradually over time before dying. Queenless colonies had no queen or evidence of a queen (presence of eggs or young brood) prior to mortality. Drone laying queen colonies possessed a queen that was laying only haploid male (drone) eggs. Laying workers were identified by the absence of a queen and the presence of many cells with multiple eggs laid on the sides of the cells. Virgin queens were determined by the presence of a queen but the absence of any eggs or young brood. Only one colony was determined to die from disease (from chalkbrood, a fungal infection). Proportion of the total was determined as the proportion of all colonies in the operation that experienced mortality for any reason.

Table 3.4 rtPCR viral and reference gene (RPS5) primer sequences.

Table 3.5 Summary of pesticides detected in forager pollen loads, 2010-12. Columns within each site are organized according to the prevalence of chemicals detected, with the most prevalent chemical detected at each site appearing first, second most common second, etc (as shown in the column, % Site Sample Detects). Class: CAH = chlorinated aromatic hydrocarbon, CYC = cyclodiene, FUNG = fungicide, HERB = herbicide, MET = metabolite, MP = monoterpene phenol, OP = organophosphate, PS = partial systemic, PYR = pyrethroid. PYRZ = pyrazole. LD_{50} = lethal dose to kill 50% of a honey bee population (contact LD_{50} used). Bee Toxicity = High $\leq$ 2 ug/bee; Moderate 2-11 ug/bee; Low > 11 ug/bee; Blanks: No data on bee toxicity or LD_{50}. Pollen Hazard Quotient (PHQ) = average ppb for each chemical/contact LD_{50}. Asterisked pesticides were those applied by the beekeeper.

3.8 Tables

Table 3.1

Model (Apiary Survival)	**Effect**	**Value**	**SE**	**df**	**T**	**P**
$\sin^{-1}(\sqrt{(\text{prop. survival})}) \sim$	Intercept	0.864	0.047	10.6	18.4	2.3×10^{-9}*
fresh pollen weight/day +	Pollen mass	0.001	0.0003	10.1	4.2	0.002*
September brood area +	Sep brood	0.318	0.056	9.96	5.6	0.0002*
September *Varroa*	Sep *Varroa*	-0.048	0.019	9.8	-2.5	0.032*
Random Effects	**Intercept**	**Variance**	**S.D.**			
	Site	0.008	0.087			
	Year	0.000	0.000			
	Residual	0.001	0.036			

Table 3.2

		Proportion of total				Proportion of total		
Site	Family	2010	2011	2012	Genera/species	2010	2011	2012
1	Apiaceae	-	0.005	-	other Apiaceae	-	0.005	-
	Asteraceae	0.317	0.554	0.295	*Artemesia*	0.010	0.107	-
					Circium	-	0.070	0.295
					Grindelia squarrosa	0.063	0.055	-
					Helianthus		0.003	-
					Sonchus	0.113	0.176	-
					Taraxacum officinale	-	0.003	-
					Tragopogon	.	0.022	-
					other Asteraceae	0.131	0.120	-
	Brassicaceae	0.060	0.107	0.042	*Brassica*	0.015	0.068	-
					other Brassicaceae	0.045	0.092	0.042
	Fabaceae	0.535	0.232	0.572	*Lathyrus*	-	0.003	-
					Medicago sativa	-	0.014	-
					Melilotus	0.468	0.118	0.074
					Phaseolus	0.007	0.020	0.106
					Trifolium	0.008	0.013	-
					Vicia	0.052	0.001	-
					other Fabaceae	-	0.010	0.392
	Poaceae	0.024	-	-	other Poaceae	0.024	-	-
	Undetermined	0.064	0.101	0.091				
2	Amarantheaceae	-	-	0.001	*Atriplex*	-	-	0.001
	Apiaceae	0.002	0.062	-	other Apiaceae	0.002	0.062	0.009
	Asteraceae	0.567	-	0.499	*Artemesia*	0.004	-	-
					Circium	0.005	-	0.229
					Helianthus	-	-	0.162
					Sonchus	0.142	-	0.024
					other Asteraceae	0.415	-	0.084
	Brassicaceae	0.134	0.072	-	*Brassica*	0.067	-	-
					Sinapsis	-	0.021	-
					other Brassicaceae	0.067	0.051	-
	Caprifoliaceae	-	-	0.010	*Lonicera*	-	-	0.010
	Cornaceae	-	-	0.013	*Cornus*	-	-	0.013
	Fabaceae	0.243	0.813	0.299	*Glycine max*	0.004	-	-
					Lathyrus	-	0.057	-
					Medicago sativa	0.014	-	-
					Melilotus	0.222	0.661	0.130
					Phaseolus	-	0.040	0.085
					Trifolium	0.004	0.054	0.051
					other Fabaceae	-	-	0.033
	Poaceae	-	-	0.015	other Poaceae	-	-	0.015
	Polemoniaceae	-	-	0.017	other Polemoniaceae	-	-	0.017
	Undetermined	0.054	0.054	0.136				
3	Amaranthaceae	0.054	0.030	-	*Atriplex*	0.013	-	-
					other Amaranthaceae	0.041	0.030	-

		Proportion of total				Proportion of total		
Site	Family	2010	2011	2012	Genera/species	2010	2011	2012
	Apiaceae	0.002	-	-	Apiaceae	0.002	-	.-
	Asteraceae	0.338	0.269	0.350	*Artemesia*	0.019	0.003	0.038
					Centaurea maculosa	-	-	0.231
					Cichorium	0.003	0.136	-
					Grindelia squarrosa	0.031	-	-
					Helianthus	-	-	0.024
					Solidago	0.002	-	-
					Sonchus	0.196	-	0.108
					Tragopogon	0.009	0.108	-
					other Asteraceae	0.078	0.022	0.091
	Brassicaceae	0.010	0.045	-	*Alyssum*	0.010	0.040	-
					other Brassicaceae	-	0.005	-
	Caryophyllaceae	0.010	0.014	-	*Silene latifolia*	0.010	0.014	-
	Chenopodaceae	-	-	0.018	other Chenopodiaceae	-	-	0.018
	Fabaceae	0.425	0.506	0.260	*Astragalus*	-	0.004	-
					Glycine max	0.020	-	-
					Lathyrus	0.018	-	-
					Lupinus	-	-	0.047
					Medicago sativa	0.014	0.007	.
					Melilotus	0.287	0.349	0.176
					Psoralea	0.017	0.007	-
					Trifolium	0.046	0.114	0.022
					Vicia	0.007	0.024	0.015
					other Fabaceae	0.017	-	-
	Lamiaceae	0.003	-	-	other Lamiaceae	0.003	-	-
	Linaceae	.	0.009	-	other Linaceae	-	0.009	-
	Polygonaceae	0.039	-	-	*Fagopyrum*	0.039	-	-
	Rosaceae	.	0.008	.	other Rosaceae	-	0.008	-
	Scrophulariaceae	0.007	-	0.079	*Linaria*	0.007	-	-
					other Scrophulariaceae	-	-	0.079
	Undetermined	0.113	0.119	0.152				
4	Amaranthaceae	0.004	-	0.167	*Atriplex*	0.004	-	0.167
	Asteraceae	0.507	0.296	0.544	*Artemesia*	0.020	-	-
					Circium	0.005	-	-
					Grindelia squarrosa	0.031	0.082	-
					Helianthus	0.106	-	-
					Solidago	0.078	-	-
					Sonchus	0.217	0.165	0.046
					other Asteraceae	0.050	0.049	0.498
	Brassicaceae	0.110	-	-	*Brassica*	-	0.123	-
					other Brassicaceae	0.110	-	-
	Caryophyllaceae	0.001	-	-	*Silene latifolia*	0.001	-	-
	Fabaceae	0.301	0.647	0.082	*Lathyrus*	-	0.024	-
					Medicago sativa	.-	0.019	-
					Melilotus	0.093	0.452	-

		Proportion of total				Proportion of total		
Site	Family	2010	2011	2012	Genera/species	2010	2011	2012
					Trifolium	0.004	-	0.082
					Vicia	0.204	0.029	-
					other Fabaceae	-	-	-
	Poaceae	0.002	-	-	other Poaceae	0.002	-	-
	Scrophulariaceae	-	-	0.107	*Linaria*	-	-	0.107
	Undetermined	0.076	0.058	0.100				
5	Asteraceae	0.440	0.226	0.080	*Artemesia*	-	0.031	-
					Circium	0.023	0.004	-
					Grindelia squarrosa	0.106	0.015	-
					Sonchus	0.260	0.152	-
					other Asteraceae	0.051	0.024	0.250
	Brassicaceae	0.103	-	-	*Brassica*	0.001	-	-
					other Brassicaceae	0.103	-	-
	Caryophyllaceae	0.003	-	-	*Silene latifolia*	0.003	-	-
	Chenopodiaceae	0.001	-	-	other Chenopodiaceae	0.001	-	-
	Fabaceae	0.340	0.517	0.312	*Astragalus*	-	0.092	-
					Lathyrus	0.003	-	-
					Medicago sativa	0.010	-	0.265
					Melilotus	0.287	0.292	0.017
					Phaseolus	0.009	-	-
					Psoralea	0.002	-	-
					Trifolium	0.013	0.093	0.030
					Vicia	0.007	0.075	-
					other Fabaceae	0.008	0.010	-
	Linaceae	-	-	-	*Linum*	0.011	-	-
	Plantaginaceae	-	-	0.155	*Plantago*	-	-	0.155
	Scrophulariaceae	-	-	0.060	*Linaria*	-	-	0.060
	Undetermined	0.101	0.212	0.223				
6	Asteraceae	0.370	0.188	0.483	*Artemesia*	0.001	0.019	-
					Centaurea maculosa	-	-	0.030
					Cichorium	-	0.027	-
					Circium	-	0.019	0.023
					Grindelia squarrosa	0.096	0.085	0.105
					Helianthus	-	-	0.025
					Liatris	0.004	-	-
					Sonchus	0.053	0.019	-
					Solidago	0.013	-	-
					other Asteraceae	0.203	0.018	0.306
	Balsaminaceae	-	0.020	-	*Impatiens*	-	0.020	-
	Brassicaceae	0.044	0.255	0.229	*Alyssum*	0.004	0.009	0.130
					Brassica	0.039	0.242	0.111
					Sinapsis	0.002	-	-
					other Brassicaceae	-	-	-
	Caprifoliaceae	-	-	0.008	*Lonicera*	-	-	0.008
	Caryophyllaceae	0.005	0.007	-	*Silene latifolia*	0.005	0.007	-
	Fabaceae	0.499	0.437	0.100	*Lathryus*	0.021	-	-

		Proportion of total				Proportion of total		
Site	Family	2010	2011	2012	Genera/species	2010	2011	2012
					Medicago sativa	0.004	-	0.006
					Melilotus	0.390	0.343	0.021
					Phaseolus	0.042	0.002	0.040
					Psoralea	-	0.002	-
					Trifolium	-	0.004	0.047
					Vicia	0.041	0.078	.-
					other Fabaceae	-	0.012	0.048
	Poaceae	-	-	0.030	other Poaceae	-	-	0.030
	Scrophulariaceae	-	0.007	-	other Scrophulariaceae	-	0.007	-
	Undetermined	0.082	0.086	0.070				

Table 3.3

Primer	Forward	Reverse
ABPV	ACCGACAAAGGGTATGATGC	CTTGAGTTTGCGGTGTTCCT
BQCV	TTTAGAGCGAATTCGGAAACA	GGCGTACCGATAAAGATGGA
CBPV	CAAAATCAACGAGCCAATCA	AGTGTGAGGATCACCGGAAC
DWV	GAGATTGAAGCGCATGAACA	TGAATTCAGTGTCGCCCATA
IAPV	CCATGCCTGGCGATTCAC	CTGAATAATACTGTGCGTATC
KBV	TGAACGTCGACCTATTGAAAAA	TCGATTTTCCATCAAATGAGC
SBV	GGGTCGAGTGGTACTGGAAA	ACACAACACTCGTGGGTGAC
RPS5	AATTATTTGGTCGCTGGAATTG	TAACGTCCAGCAGAATGTGGTA

Table 3.4

Site	Residue	Pollen Hazard Quotient	Contact LD50 (ug/bee)	Class	High (ppb)	Low (ppb)	Median (ppb)	Mean (ppb)	No. detects (6 total) 2010	No. detects (5 total) 2011	No. detects (3 total) 2012	One sample, or one year only
1	Bifenthrin	1220.00	0.01	PYR				12.20	-	1	-	8/8/11
	Cyhalothrin total	909.00	0.05	PYR	88.9	2	45.45	45.45	-	2	-	2011
	Esfenvalerate	260.00	0.02	PYR				5.20	1	-	-	8/17/10
	Chlorpyrifos	136.83	0.06	OP	21.7	3	7.1	8.21	-	5	2	
	Fluvalinate*	21.43	3.5	PYR	281	2.5	20.3	75.00	6	1	1	
	Fenpyroximate	4.18	11	PYRZ	185	7.7	34	46.00	3	1	-	
	Chlorothalonil	4.01	142.38	PYR	807	336	571.5	571.50	1	1	-	
	Coumaphos*	1.26	22	OP	85.6	1.1	17.6	27.80	4	3	1	
	Pendimethalin	0.60	49.8	HERB				29.70	-	-	1	6/21/10
	Carbendazim (MBC)	0.13	50	FUNG	7.1	trace	6.3	6.30	2	-	1	
	Thymol*			MP	11,500	trace	3,215.00	4355.90	6	2	1	
	DMPF*			MET	392	trace	92.7	107.01	4	1	1	
	Para-dichlorobenzene*			CAH	518	172	345	345.00	-	2	-	2011
	Coumaphos oxon*			MET	8.5	8.5	8.5	8.50	-	-	1	
2	Bifenthrin	1420.00	0.01	PYR				14.20	-	-	1	8/8/11
	Chlorpyrifos	166.33	0.06	OP	10.6	trace	8.4	9.98	-	3	3	
	Cyhalothrin total	88.60	0.05	PYR	6.9	3.4	4	4.43	-	2	1	
	Chlorothalonil	7.87	142.38	PYR				1120.00	1	-	-	6/21/10
	Fluvalinate*	7.17	3.5	PYR	81.8	5.5	14.3	25.08	6	-	-	2010
	Fenpyroximate	1.98	11	PYRZ				21.80	1	-	-	6/21/10
	Coumaphos*	0.40	22	OP	21.4	3.7	10.2	8.82	3	-	1	

Site	Residue	Pollen Hazard Quotient	Contact LD50 (ug/bee)	Class	High (ppb)	Low (ppb)	Median (ppb)	Mean (ppb)	No. detects (6 total) 2010	No. detects (5 total) 2011	No. detects (3 total) 2012	One sample, or one year only
	Carbendazim (MBC)	0.18	50	FUNG	9.6	trace	8.75	8.75	2	-	1	
	Tebuconazole	0.04	200	FUNG				8.50	-	1	-	7/9/11
	Thymol*			MP	5010	170	469	1266.00	6	-	1	
	DMPF*			MET	50.7	13.7	18.5	27.63	3	-	-	2010
	Para-dichlorobenzene*			CAH	404	139	181	241.30	-	3	-	2011
3	Bifenthrin	630.00	0.01	PYR				6.30	-	1	-	9/18/11
	Esfenvalerate	205.00	0.02	PYR				4.10	1	-	-	9/8/10
	Chlorpyrifos	201.33	0.06	OP	25.8	1.8	13.2	12.08	2	5	3	
	Cyhalothrin total	46.00	0.05	PYR				2.30	-	1	-	9/18/11
	Fluvalinate*	7.17	3.5	PYR	62.8	8.3	18.4	25.08	5	-	1	
	Methyl Parathion	5.64	1.45	OP				8.20	1	-	-	6/10/10
	Coumaphos*	0.33	22	OP	14	2.2	2.3	7.23	2	1	1	
	Carbendazim (MBC)	0.13	50	FUNG	6.3	trace	6.3	6.30	2	-	-	2010
	Trifluralin	trace	46.34	HERB	trace	trace	trace	trace	-	-	1	8/1/12
	Thymol*			MP	4290	176	372	768.33	5	4	-	
	DMPF*			MET	30.6	7.5	15	21.53	3	1	-	
	Para-dichlorobenzene*			CAH	394	149	180	241.00	-	3	-	2011
4	Bifenthrin	2360.00	0.01	PYR				23.60	1	0	0	8/17/10
	Chlorpyrifos	1392.50	0.06	OP	490	7.1	32.5	83.55	3	3	2	
	Esfenvalerate	1160.00	0.02	PYR				23.20	1	0	0	8/1/10
	Cypermethrin	796.67	0.03	PYR	29.6	18.2	23.9	23.90	2	0	0	2010
	Cyfluthrin	633.33	0.03	PYR	32.2	5.8	19	19.00	2	0	0	2010
	Cyhalothrin total	386.40	0.05	PYR	52.6	4.2	7.9	19.32	3	2	-	

Site	Residue	Pollen Hazard Quotient	Contact LD50 (ug/bee)	Class	High (ppb)	Low (ppb)	Median (ppb)	Mean (ppb)	No. detects (6 total) 2010	No. detects (5 total) 2011	No. detects (3 total) 2012	One sample, or one year only
	Fenpyroximate	6.11	11	PYRZ				67.20	1	-	-	6/21/10
	Fluvalinate*	4.75	3.5	PYR	142	2.6	7.1	16.63	6	-	1	
	Coumaphos*	2.93	22	OP				64.40	1	-	-	6/21/10
	Endosulfan sulfate	1.21	7.02	CYC				8.50	1	-	-	8/1/10
	Pyraclostrobin	1.11	100	FUNG				111.00	1	-	-	8/17/10
	Endosulfan II	1.04	7.02	CYC				7.30	1	-	-	8/1/10
	Chlorothalonil	0.38	142.38	PYR				54.00	1	-	-	7/19/10
	Trifluralin	0.37	46.34	HERB				17.20	1	-	-	8/17/10
	Trifloxystrobin	0.32	193.75	FUNG				62.20	-	-	1	7/13/12
	Carbendazim (MBC)	trace	50	FUNG	trace	trace	trace	trace	2	0	0	2010
	Thymol*			MP	25400	trace	230	2552.38	6	1	1	
	DMPF*			MET	83.1	11	23.8	35.43	1	1	-	
	Para-dichlorobenzene*			CAH				201.00	-	1	-	8/26/11
5	Cypermethrin	410.00	0.03	PYR				12.30	-	1	1	6/21/10
	Esfenvalerate	390.00	0.02	PYR				7.80	1	-	-	6/21/10
	Chlorpyrifos	324.00	0.06	OP	59.2	1.9	17.5	19.44	-	5	3	
	Cyfluthrin	196.67	0.03	PYR				5.90	1	-	-	6/21/10
	Cyhalothrin total	74.00	0.05	PYR				3.70	-	1	-	8/8/11
	Methyl Parathion	7.45	1.45	OP				10.80	1	-	-	6/21/10
	Fluvalinate*	4.75	3.5	PYR	45.2	13.3	19	16.63	6	1	-	
	Fenpyroximate	2.26	11	PYRZ	28.6	19.2	23	24.85	2	-	1	
	Coumaphos*	0.44	22	OP	25.7	1	8.8	9.63	4	2	2	
	Carbendazim	0.12	50	FUNG	6.3	5.7	5.9	5.90	2	-	-	2010

Site	Residue	Pollen Hazard Quotient	Contact LD50 (ug/bee)	Class	High (ppb)	Low (ppb)	Median (ppb)	Mean (ppb)	No. detects (6 total) 2010	No. detects (5 total) 2011	No. detects (3 total) 2012	One sample, or one year only
	(MBC)											
	Thymol*			MP	3970	trace	613	730.56	5	3	3	
	DMPF*			MET	44.2	6.9	17.3	24.00	5	2	2	
	Para-dichlorobenzene*			CAH	150	133	137	140.00	-	3	-	2011
6	Deltamethrin	4900.00	0.04	PYR				196.00	1	-	-	8/17/10
	Esfenvalerate	530.00	0.02	PYR				10.60	1	-	-	8/17/10
	Cyfluthrin	413.33	0.03	PYR				12.40	1	-	-	8/17/10
	Chlorpyrifos	200.00	0.06	OP	22.7	3.7	8.6	12.00	-	5	2	
	Cyhalothrin total	65.00	0.05	PYR	4.3	2.8	3.6	3.25	1	1	-	
	Fenpyroximate	25.09	11	PYRZ				276.00	1	-	-	6/21/10
	Chlorfenapyr	18.57	0.14	PS				2.60	1	-	-	8/17/10
	Fluvalinate*	11.98	3.5	PYR	258	7.8	12.5	41.93	6	1	-	
	Coumaphos*	1.20	22	OP	120	4.1	6.6	26.43	3	2	-	
	Carbendazim (MBC)	0.20	50	FUNG	12.6	trace	10.2	10.15	2	2	-	
	Chlorothalonil	0.18	142.38	PYR				26.00	1	-	-	6/21/10
	Oxyfluorfen	0.04	100	HERB				4.00	1	-	-	8/17/10
	Vinclozolin	0.01	113.67	FUNG				1.00	1	-	-	8/17/10
	Thymol*			MP	44,800	81	216	3536.00	5	4	1	
	DMPF*			MET	49.7	trace	29	30.60	3	2	-	
	Para-dichlorobenzene*			CAH	208	144	161	171.00	-	3	-	2011

3.9 Figure legends

Figure 3.1 Colony adult population size (mean number of combs covered in adult bees ± s.e., n=24 colonies per site) by date and site, 2010-2012.

Figure 3.2 Colony pupal brood population (mean number of frames of pupating brood ± s.e., n=24 colonies per site) by date and site, 2010-2012.

Figure 3.3 Colony pollen stores (mean number combs of stored pollen ± s.e., n=24 colonies per site) by date and site, 2010-2012.

Figure 3.4 Pollen families and common genera detected (background chart colors), average colony pollen collection per day (dashed line) over each summer. Proportion (primary y-axis) of pollen families and commonly occurring genera are represented by the background colors and legend in each graph. Families/genera are depicted from bottom to top and left to right on each graph. Average amount (grams) of pollen collected per 24 hours (secondary y-axis) in 3 colony traps per apiary is represented by the dashed line. For example, on June 21, 2010 at site 1, colonies collected ~60 g of pollen on average which was composed of approximately 80% Fabaceae, 18% Brassicaceae, and 2% undetermined pollen.

Figure 3.5 Pollen diversity analyses by family (6a and 6b) and genus (6c and 6d). A total of 15 families and 28 genera were identified in forager bee-collected pollen pellets trapped across all six sites and three years (2010-2012). Shannon-Weiner index (6a and 6c) and Jaccard similarity (6b and 6d) were determined for both family and genus diversity.

Figure 3.6 Queen status of colonies by year (2010-12) and site (1-6), May-March of each year. Queen status depicted occurred on the sample date immediately prior to the colony being reported as dead. 7a depicts the status of all colonies as a proportion of the whole (n=24 colonies per site), while 7b depicts the status (number of dead colonies) of just the colonies that died. Green denotes surviving colonies, red-scale denotes colonies with queen problems, blue-scale denotes queen-right colonies that died from other (non-queen) issues.

Figure 3.7 Colony *Varroa destructor* mite levels (mean % mite infestation ± s.e., n=24 colonies per site) by date and site, 2010-2013. Y-axis is adjusted up for 2012 relative to 2010 and 2011 to account for greater infestation levels in that year.

Figure 3.8 Colony *Nosema* spp. infection levels (mean spores per bee ± s.e., n=24 colonies per site) by date and site, 2010-2013.

3.10 Figures

Figure 3.1

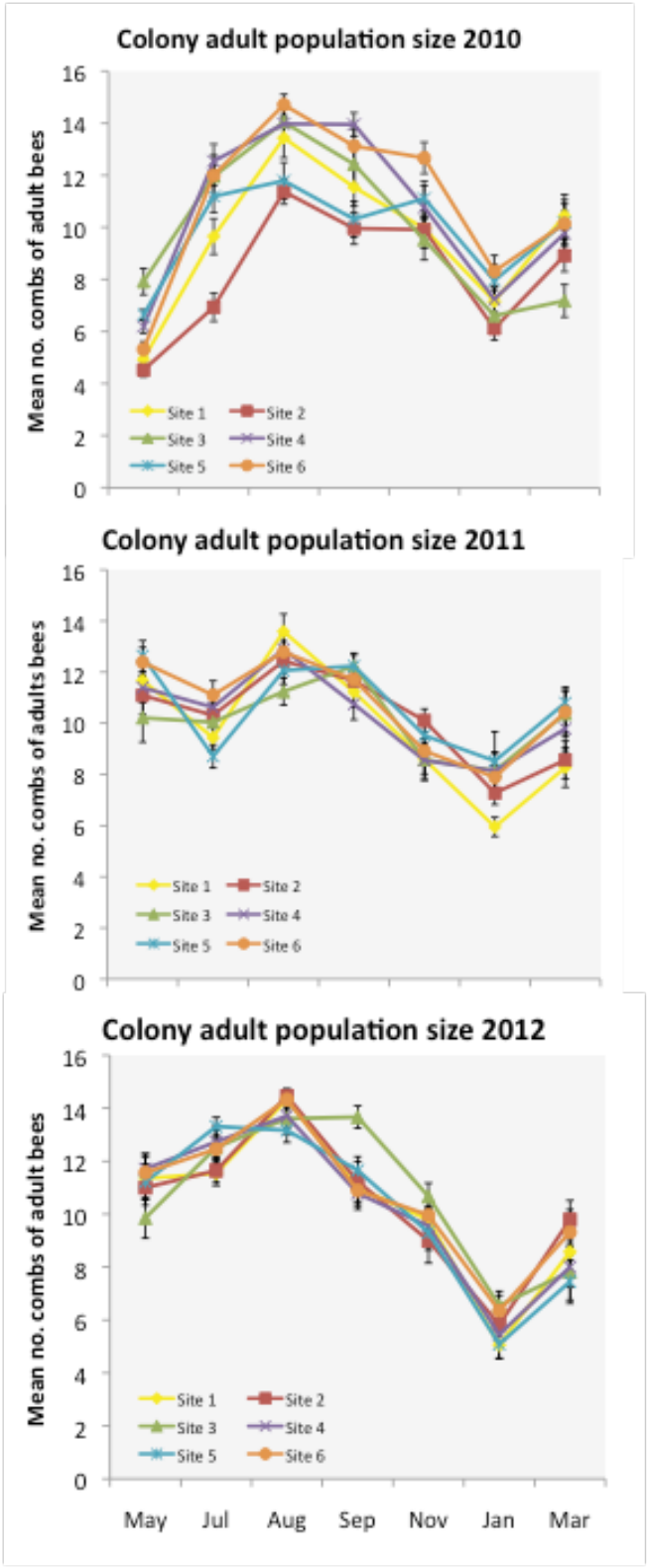


Figure 3.2

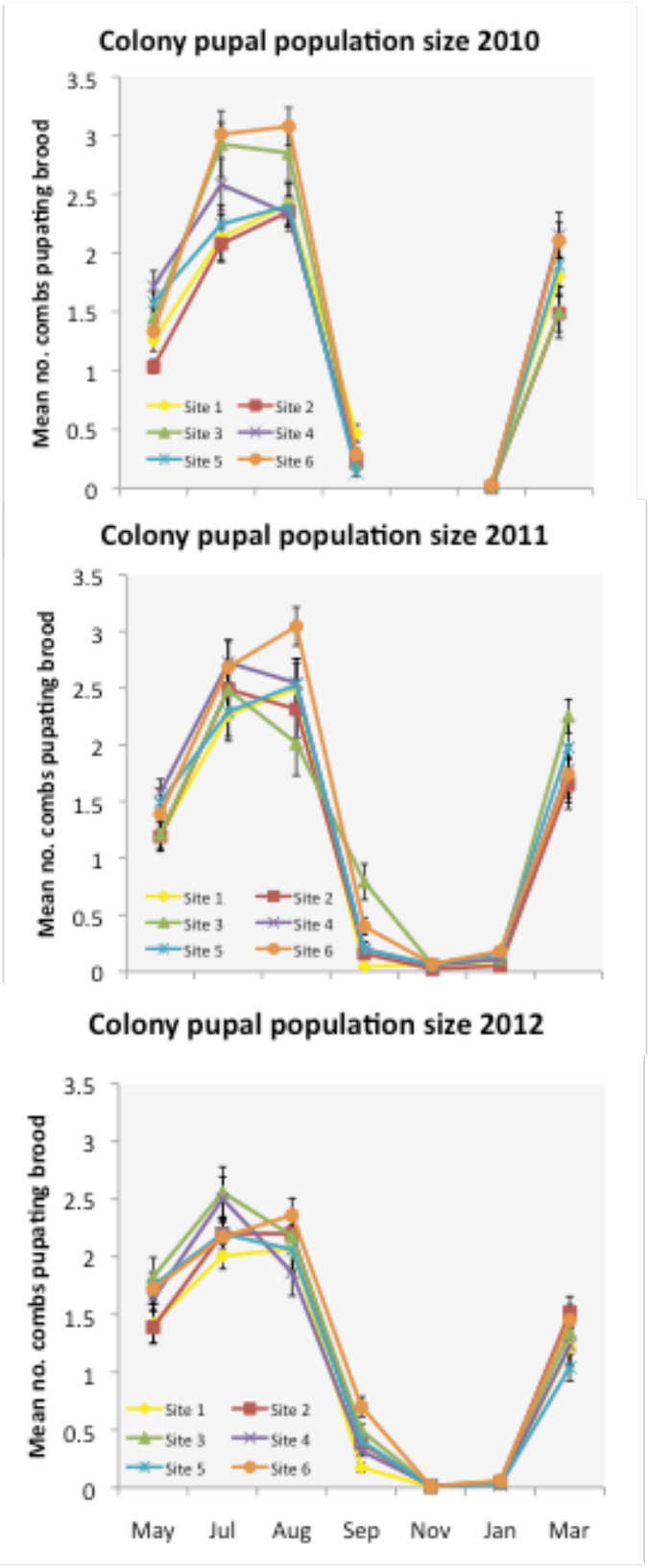

Colony pupal population size 2010
Mean no. combs pupating brood
3.5
3
2.5
2
1.5
1
0.5
0
Site 1
Site 2
Site 3
Site 4
Site 5
Site 6
Colony pupal population size 2011
Mean no. combs pupating brood
3.5
3
2.5
2
1.5
1
0.5
0
Site 1
Site 2
Site 3
Site 4
Site 5
Site 6
Colony pupal population size 2012
Mean no. combs pupating brood
3.5
3
2.5
2
1.5
1
0.5
0
Site 1
Site 2
Site 3
Site 4
Site 5
Site 6
May
Jul
Aug
Sep
Nov
Jan
Mar

Figure 3.3

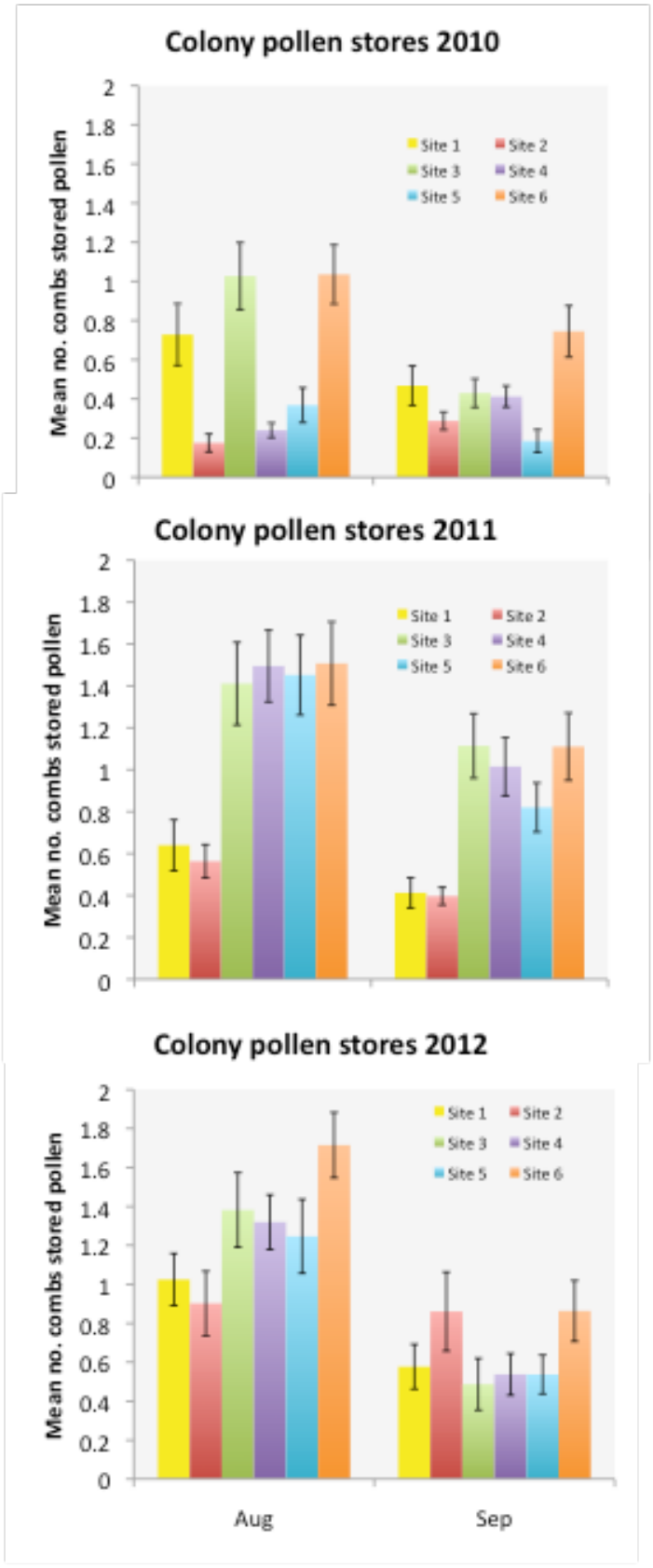
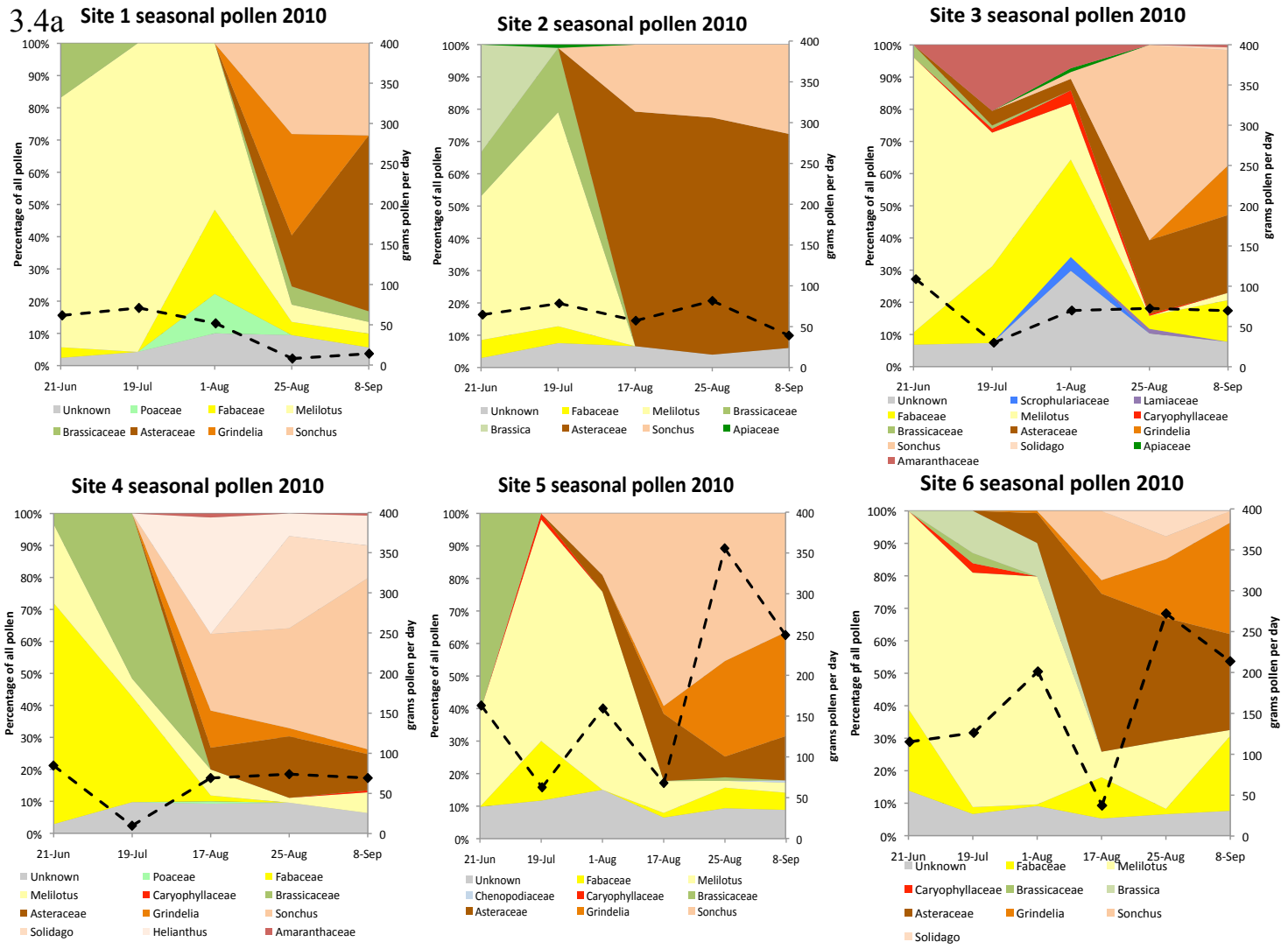
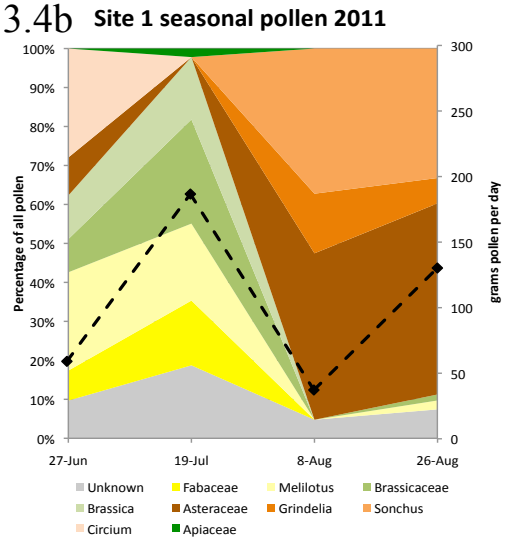

Colony pollen stores 2010
Mean no. combs stored pollen
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Site 1
Site 2
Site 3
Site 4
Site 5
Site 6
Colony pollen stores 2011
Mean no. combs stored pollen
Site 1
Site 2
Site 3
Site 4
Site 5
Site 6
Colony pollen stores 2012
Mean no. combs stored pollen
Site 1
Site 2
Site 3
Site 4
Site 5
Site 6
Aug
Sep

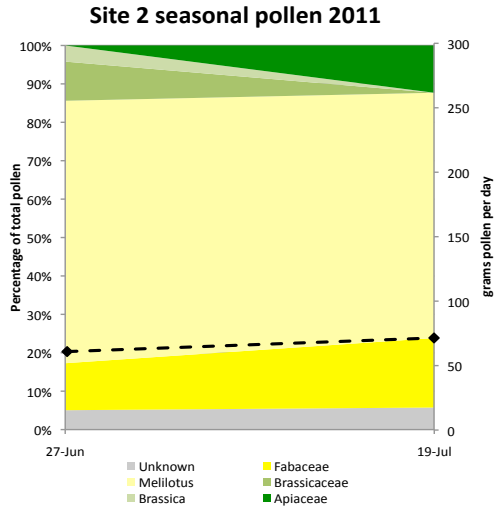
Figure 3.4

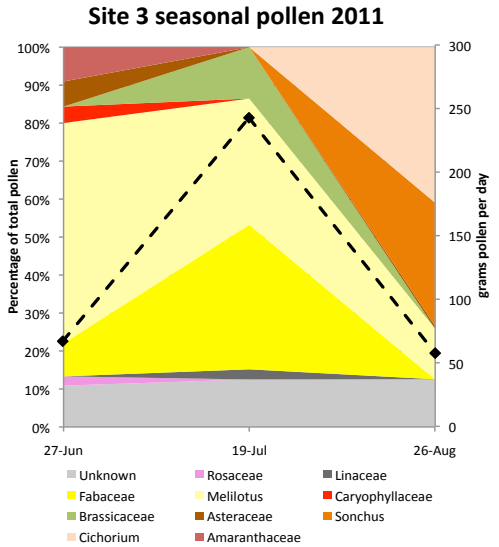
3.4a

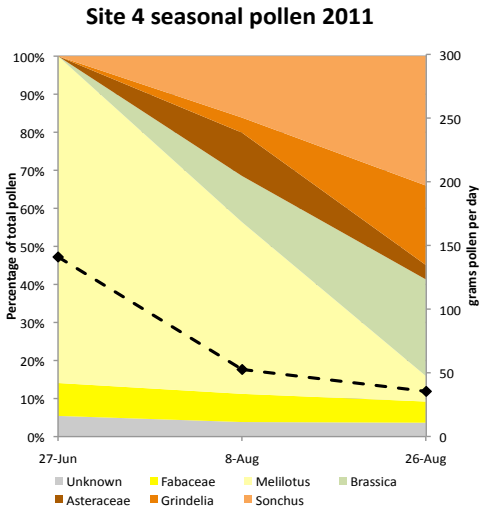


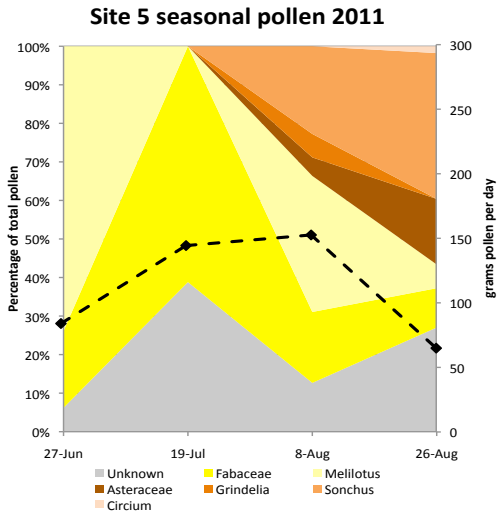
3.4b

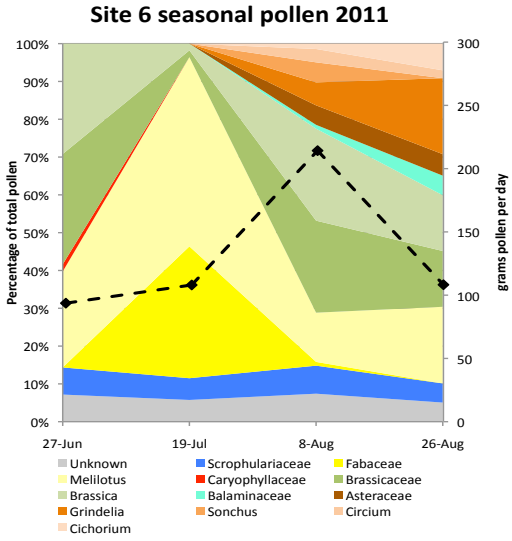

Site 1 seasonal pollen 2011
Percentage of all pollen
grams pollen per day
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
300
250
200
150
100
50
0
27-Jun
19-Jul
8-Aug
26-Aug
Unknown
Fabaceae
Melilotus
Brassicaceae
Brassica
Asteraceae
Grindelia
Sonchus
Circium
Apiaceae


Site 2 seasonal pollen 2011
Percentage of total pollen
grams pollen per day
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
300
250
200
150
100
50
0
27-Jun
19-Jul
Unknown
Fabaceae
Melilotus
Brassicaceae
Brassica
Apiaceae

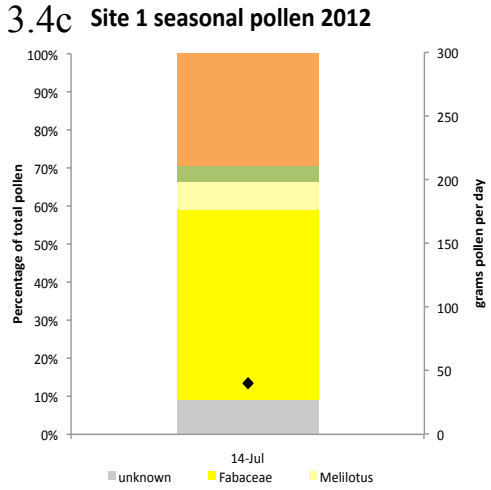

Site 3 seasonal pollen 2011
Percentage of total pollen
grams pollen per day
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
300
250
200
150
100
50
0
27-Jun
19-Jul
26-Aug
Unknown
Rosaceae
Linaceae
Fabaceae
Melilotus
Caryophyllaceae
Brassicaceae
Asteraceae
Sonchus
Cichorium
Amaranthaceae

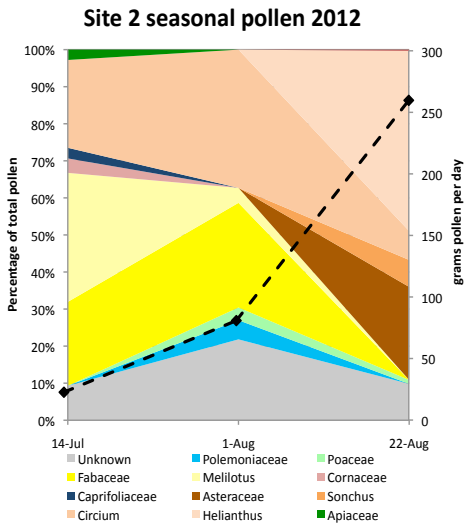

Site 4 seasonal pollen 2011
Percentage of total pollen
grams pollen per day
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
300
250
200
150
100
50
0
27-Jun
8-Aug
26-Aug
Unknown
Fabaceae
Melilotus
Brassica
Asteraceae
Grindelia
Sonchus

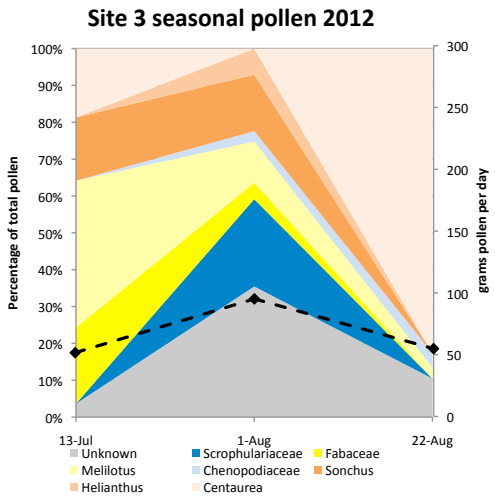

Site 5 seasonal pollen 2011
Percentage of total pollen
grams pollen per day
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
300
250
200
150
100
50
0
27-Jun
19-Jul
8-Aug
26-Aug
Unknown
Fabaceae
Melilotus
Asteraceae
Grindelia
Sonchus
Circium

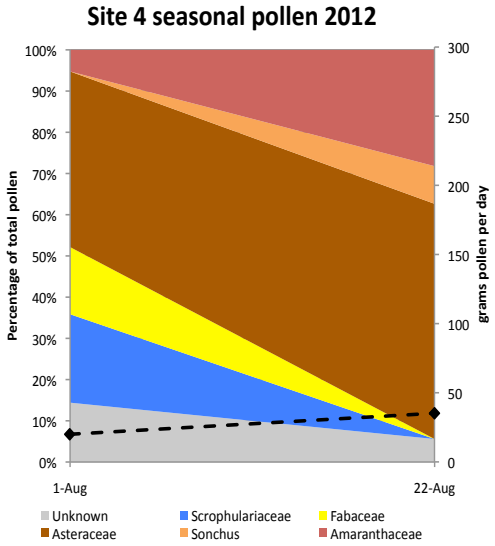

Site 6 seasonal pollen 2011
Percentage of total pollen
grams pollen per day
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
300
250
200
150
100
50
0
27-Jun
19-Jul
8-Aug
26-Aug
Unknown
Scrophulariaceae
Fabaceae
Melilotus
Caryophyllaceae
Brassicaceae
Brassica
Balaminaceae
Asteraceae
Grindelia
Sonchus
Circium
Cichorium

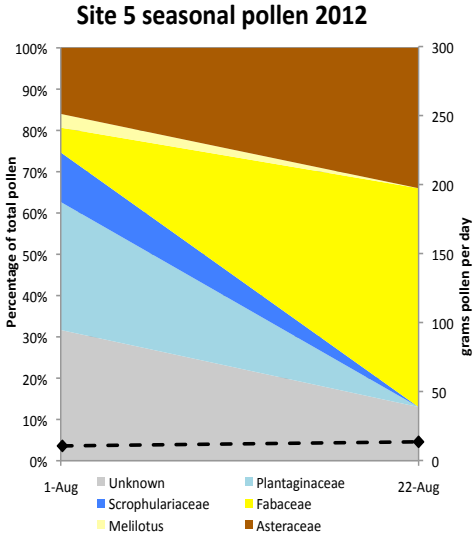
3.4c


Site 1 seasonal pollen 2012
Percentage of total pollen
grams pollen per day
14-Jul
unknown
Fabaceae
Melilotus


Site 2 seasonal pollen 2012
Percentage of total pollen
grams pollen per day
14-Jul
1-Aug
22-Aug
Unknown
Polemoniaceae
Poaceae
Fabaceae
Melilotus
Cornaceae
Caprifoliaceae
Asteraceae
Sonchus
Circium
Helianthus
Apiaceae


Site 3 seasonal pollen 2012
Percentage of total pollen
grams pollen per day
13-Jul
1-Aug
22-Aug
Unknown
Scrophulariaceae
Fabaceae
Melilotus
Chenopodiaceae
Sonchus
Helianthus
Centaurea


Site 4 seasonal pollen 2012
Percentage of total pollen
grams pollen per day
1-Aug
22-Aug
Unknown
Scrophulariaceae
Fabaceae
Asteraceae
Sonchus
Amaranthaceae


Site 5 seasonal pollen 2012
Percentage of total pollen
grams pollen per day
1-Aug
22-Aug
Unknown
Plantaginaceae
Scrophulariaceae
Fabaceae
Melilotus
Asteraceae

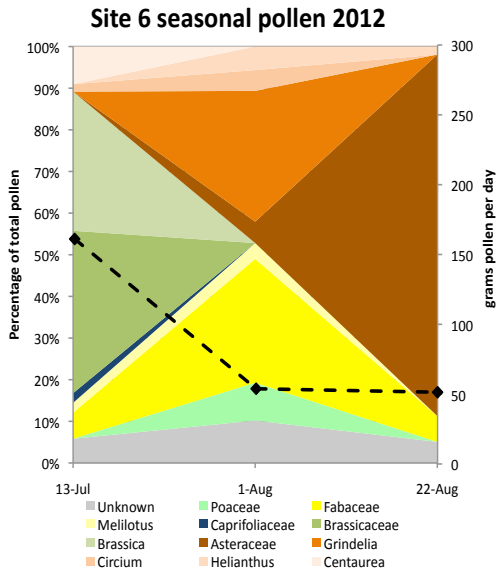
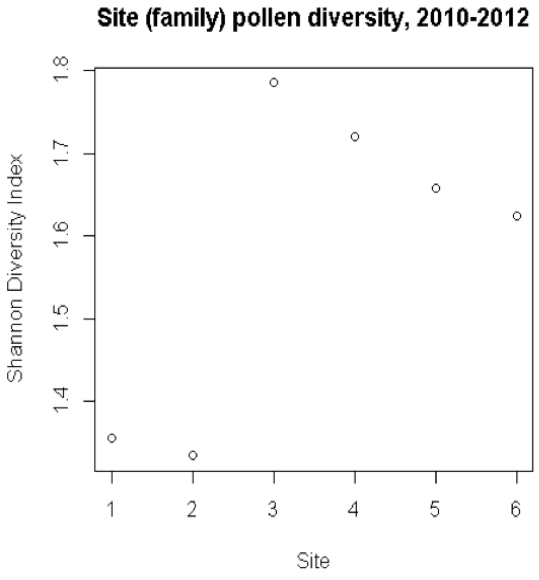
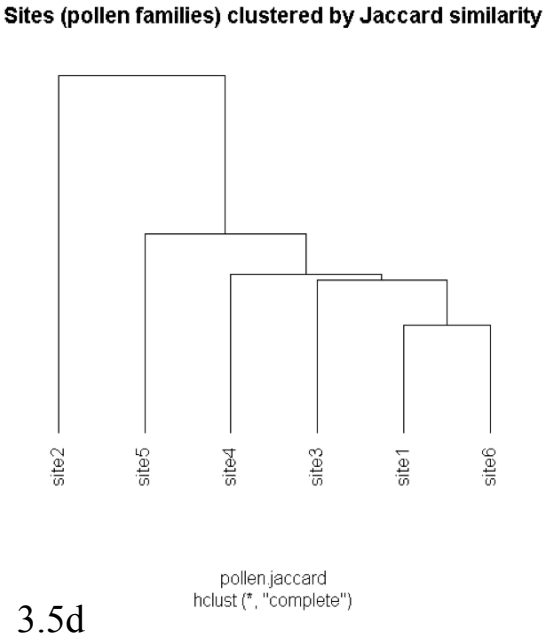

Site 6 seasonal pollen 2012
Percentage of total pollen
grams pollen per day
13-Jul
1-Aug
22-Aug
Unknown
Poaceae
Fabaceae
Melilotus
Caprifoliaceae
Brassicaceae
Brassica
Asteraceae
Grindelia
Circium
Helianthus
Centaurea

Figure 3.5

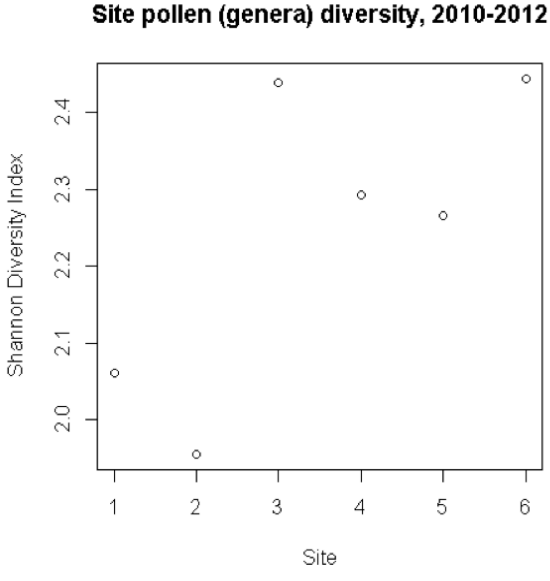
3.5a



3.5b



3.5c



3.5d

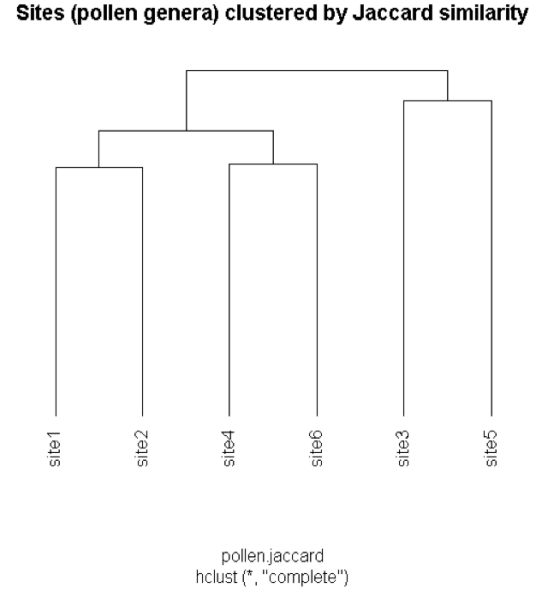


Figure 3.6

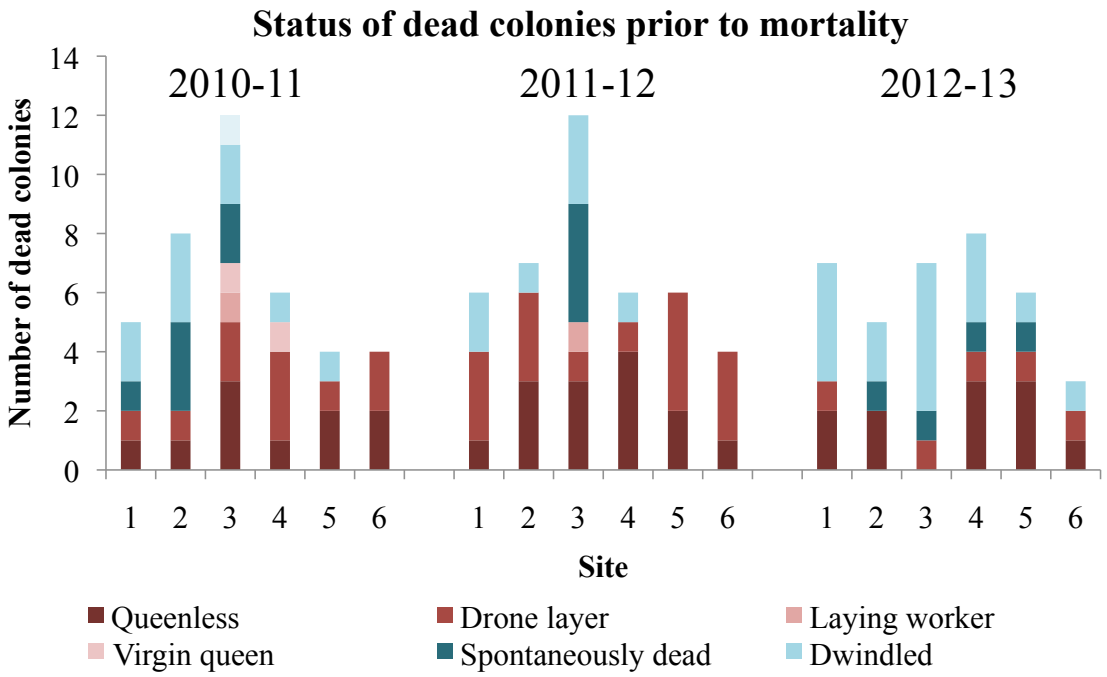

Status of dead colonies prior to mortality
2010-11
2011-12
2012-13
Number of dead colonies
0
2
4
6
8
10
12
14
1 2 3 4 5 6
1 2 3 4 5 6
1 2 3 4 5 6
Site
Queenless
Drone layer
Laying worker
Virgin queen
Spontaneously dead
Dwindled

Figure 3.7

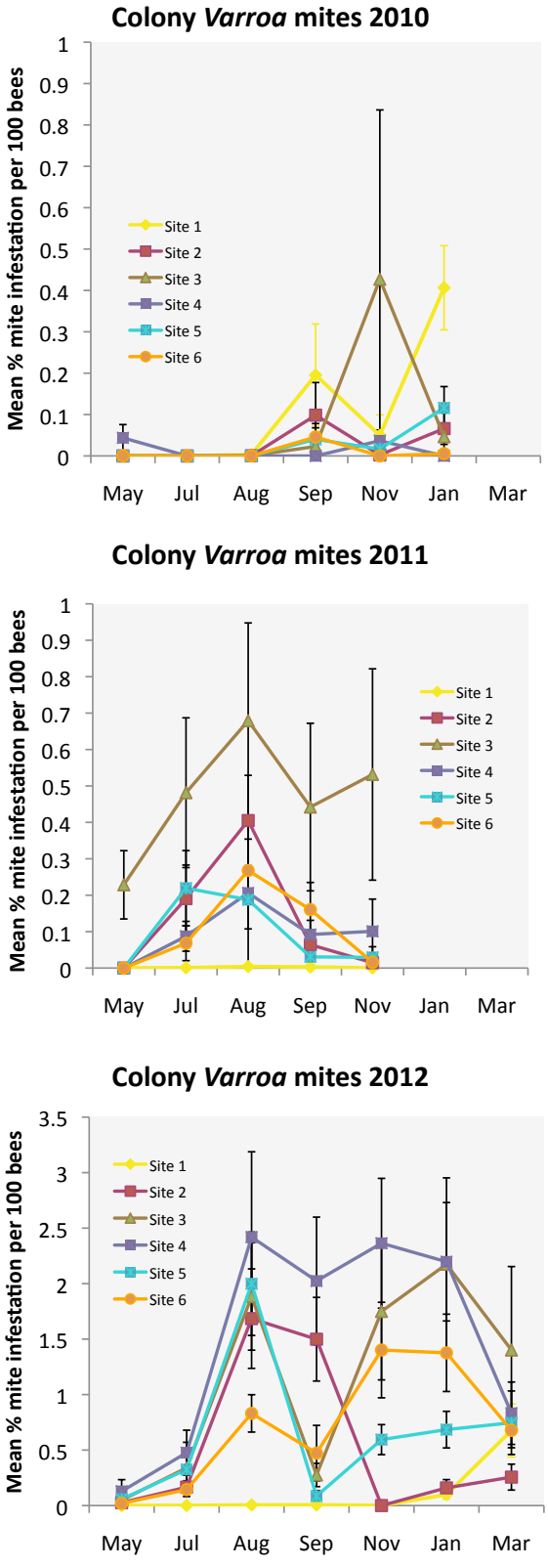
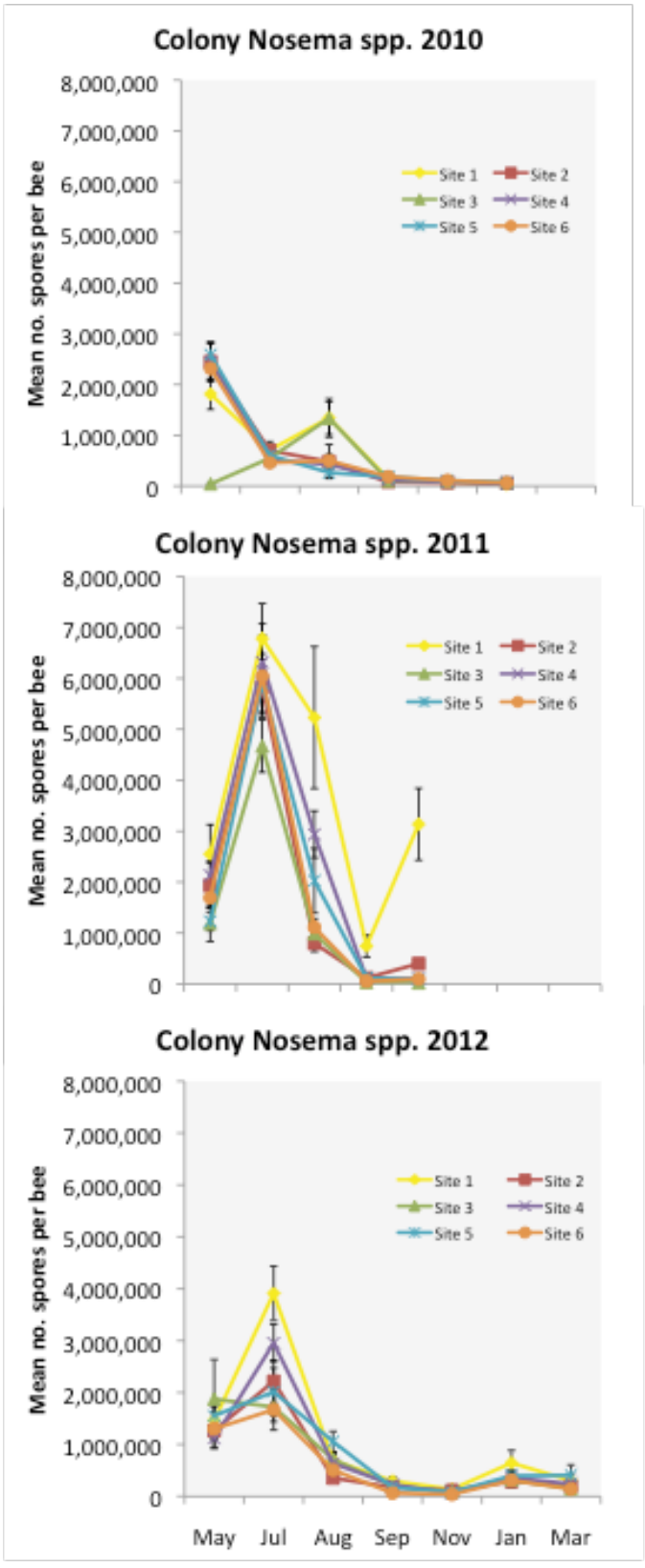

Colony *Varroa* mites 2010
Mean % mite infestation per 100 bees
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Site 1
Site 2
Site 3
Site 4
Site 5
Site 6
May
Jul
Aug
Sep
Nov
Jan
Mar
Colony *Varroa* mites 2011
Mean % mite infestation per 100 bees
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Site 1
Site 2
Site 3
Site 4
Site 5
Site 6
May
Jul
Aug
Sep
Nov
Jan
Mar
Colony *Varroa* mites 2012
Mean % mite infestation per 100 bees
3.5
3
2.5
2
1.5
1
0.5
0
Site 1
Site 2
Site 3
Site 4
Site 5
Site 6
May
Jul
Aug
Sep
Nov
Jan
Mar

Figure 3.8


Colony Nosema spp. 2010
Mean no. spores per bee
8,000,000
7,000,000
6,000,000
5,000,000
4,000,000
3,000,000
2,000,000
1,000,000
0
Site 1
Site 2
Site 3
Site 4
Site 5
Site 6
Colony Nosema spp. 2011
Mean no. spores per bee
8,000,000
7,000,000
6,000,000
5,000,000
4,000,000
3,000,000
2,000,000
1,000,000
0
Site 1
Site 2
Site 3
Site 4
Site 5
Site 6
Colony Nosema spp. 2012
Mean no. spores per bee
8,000,000
7,000,000
6,000,000
5,000,000
4,000,000
3,000,000
2,000,000
1,000,000
0
Site 1
Site 2
Site 3
Site 4
Site 5
Site 6
May
Jul
Aug
Sep
Nov
Jan
Mar

CHAPTER 4

Individual bee analysis of the effects of land use on honey bee health and survival

Matthew Smart

4.1 Introduction

Previous chapters of this dissertation examined 1) the influence of land use on the survival of commercial honey bee colonies and statistical modeling of annual colony survival based on land use (Chapter 2), and 2) the ability of *colony* measures to predict survival (Chapter 3). This chapter will examine measures taken from *individual bees* within the same North Dakota apiaries, and the ability of those measures to represent the annual survivorship of colonies across the six apiaries.

Honey bees are dependent on the availability and collection of environmental pollen for the amino acid, lipid, vitamin, and mineral components of their diet (Brodschneider and Crailsheim, 2010). The same 10 essential amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) required in the diets of vertebrates are likewise needed by honey bees (de Groot 1953), which they acquire via direct pollen-feeding, consumption of bee bread, and/or via trophallaxis. Colonies maintain a modest yet consistent store of pollen in the hive throughout the growing season (around 1 kg), enough to last for approximately one week in the event of a pollen dearth (Seeley, 1995). In-hive demand for pollen, given existing colony stores, remains relatively stable over the summer, despite considerable fluctuation in the environmental availability of pollen due to plant phenology and weather conditions (Seeley, 1995).

Pollen balance in the hive is central to colony growth and sustainability, and effects many downstream processes such as brood-rearing and behavioral development of bees. Protein balance in individual bees affects maturation and longevity, overwintering, and nutritional physiology and immunity such as the size and protein content of the hypopharyngeal glands (Huang et al., 1994; Crailsheim, 1990; Sagili and Pankiw, 2007; DeGrandi-Hoffman et al., 2010), abdominal lipid stores (Toth et al., 2005; Alaux et al., 2010a), levels of vitellogenin (Vg) an important storage protein (Alaux et al., 2011; Nilsen et al., 2011; Di Pasquale et al., 2013), oxidative stress resistance (Amdam et al., 2004b), and the immune response (Amdam et al., 2004b; Amdam et al., 2005a; Alaux et al., 2010a).

The switch in diet that occurs as bees age (around 6-16 days post-eclosion), wherein bees consume less pollen, is also associated with many physiological and behavioral developmental changes in the worker honey bee including: increasing juvenile hormone (JH) titers and a concurrent decline in hemolymph levels of Vg, a general decline in immune competence, and the switch to riskier "outside tasks" such as guarding the colony entrance, embarkation on orientation flights, initiation of foraging behavior, and ultimately, senescence (Huang and Robinson, 1996; Amdam and Omholt, 2002; Amdam et al., 2003; Amdam et al., 2004b; Amdam et al., 2005a; Nelson et al., 2007; Amdam et al., 2011). Interactions between Vg, JH, and bee nutritional state, including signaling molecules such as insulin-like peptides (ilps), are thought to participate in the behavioral maturation and longevity of the honey bee (Corona et al., 2007). Importantly, other researchers have shown that abdominal expression of both Vg and ilp-1 (as opposed

to brain expression as in Corona et al., 2007) increase during amino acid supplementation (Nilsen et al., 2011).

Vitellogenin in particular plays multiple roles in honey bee nutrition, immunity, stress resistance, behavioral development, ageing, and longevity (Amdam and Omholt 2002; Seehuus et al., 2006; Marco Antonio et al., 2008; Munch et al., 2008; Nilsen et al., 2011). In honey bee workers Vg is the most common protein found circulating in the hemolymph, though it is also found in the fat body and hypopharyngeal glands of workers (Fluri et al., 1982; Amdam et al., 2011). Workers eclosing in late fall that are exposed to declining larval rearing and foraging activities are known as overwintering diutinous bees. These long-lived and stress-resilient individuals accumulate relatively high hemolymph and fat body Vg titers throughout the winter (Amdam and Omholt, 2002; Amdam et al., 2011).

Protein and fat production, including Vg, primarily occur in the insect fat body. This invertebrate organ has been analogized to the vertebrate liver and adipose tissue, exerting effects on both nutrition and immunity (Wheeler and Robinson, 2014). The organ exists as a single cell layer, lining the inner surface of the abdomen (Amdam et al., 2011). The relative mass of the insect fat body (lipids) has been previously used as an indirect proxy for age and nutritional state (Toth et al., 2005; Alaux et al., 2010a; Ament et al. 2011), immunocompetence (Wilson-Rich et al., 2008) and longevity (Ellers, 1996; Duoms et al., 2002).

In terms of immunity, honey bees as social insects may be described as possessing multiple layers of immunity, including those at the colony (super-organismal), individual bee (organismal), and genetic levels (Wilson-Rich et al., 2009). The various, sequential

levels of immunity may be thought of as layers of an onion, beginning with the outermost layer represented by the exterior walls of the colony itself, followed by the cuticles and epithelia of individual bees, and finally the individual cellular and humoral immune defenses; each subsequent layer is more specific in its target than the previous. An individual bee's immune system is fine tuned to specific types of pathogen and parasite challenge and is composed of both the cellular and humoral arms of the immune system.

The cellular immune response is accomplished via freely circulating hemocytes that are tasked with recognizing and neutralizing large foreign bodies such as parasites and aggregations of bacteria in the hemolymph. Binding of hemocytes leads to the aggregation, encapsulation, and asphyxiation or starvation of the pathogen(s); a process often aided by the production and secretion of phenoloxidase (PO) leading to melanogenesis of the foreign body by the hemocytes themselves (see chapter 1 for further details).

In contrast, the humoral immune response is inducible, but also relies upon pattern recognition receptors present in the blood of honey bees, including proteins that recognize and bind to lipopolysaccharides and ß-1,3 glucans of bacteria and fungi, respectively (Marmaras and Lampropoulou. 2009). Binding of such recognition proteins to pathogens in the hemolymph leads to cascades of intracellular protein activation and effector nuclear transcription and subsequent protein translation within the fat body. Depending on the class of PAMP present, several over-lapping pathways (Toll, IMD, JAK/STAT, JNK) may be triggered leading to the production of anti-microbial peptides, melanization, proliferation of hemocytes, and/or cell apoptosis (Tzou et al., 2002;

Hoffmann, 2003; Evans et al., 2006; Marmaras and Lampropoulou. 2009; Gonzalez-Santoyo and Cordoba-Aguilar 2011).

Antimicrobial molecules responsible for the innate humoral immune response in insects are called antimicrobial peptides (AMPs). The activity of these peptides and polypeptides is thought to be quite broad, killing many strains of bacteria and fungi while having relatively low toxicity to the host organism (Hetru et al. 1998). Defensins are thought to act by disrupting the permeability of the bacterial membrane (Klowden 2007) that leads to a loss of cytoplasmic ATP and inhibition of respiration (Cociancich et al. 1993; Bulet et al. 1999). Abaecin is most active against gram-negative bacteria, but instead of disrupting the bacterial membrane, these residues are thought to bind to bacterial proteins, thus taking longer than defensins to kill (6-12hr) (Klowden 2007). Finally, hymenoptaecin is primarily active against gram-negative bacteria. Hymenoptaecin is thought to increase the permeability of the outer and inner membrane of invading bacteria (Bulet et al. 1999; Klowden 2007).

Additionally, stimulation of humoral immune pathways are thought to trigger the production of lysozymes (1-3) in honey bees (Evans et al., 2006); important enzymes involved in responding to both bacteria and fungi, and possibly promoting the expression of other AMPs (Imler and Bulet, 2005; Evans, 2006). Lysozyme activity against bacteria is achieved via cleavage of peptidoglycan bonds in the cell wall (Meister et al., 1997). Further, lysozyme-2 specifically has been shown to be upregulated in response to infection by the bacteria, *P. larvae*, and the fungus, *A. apis* in honey bees (Evans, 2006).

The aim of this study was to determine the nutritional and immunological responses of individual nurse bees (7-day- old adults) originating from a subset of colonies located in sites exposed to varying agricultural land use (the six study sites previously analyzed in chapters 2 and 3). The specific goal was to determine individual nurse bee measures that varied across the study sites and times of year that are predictive of annual colony survival. It was not feasible to analyze individual bees from every colony from each site; thus a subset of colonies (six per site) was sampled in this part of the study, and the measured variables were used to represent the site.

4.2 Materials and methods

4.2.1 Collection of nurse bees

At each sampling interval, six of the 24 colonies per apiary were selected for analysis of individual bee nutrition and immune levels. On each sample date, one colony per pallet was chosen at each site (for a total of six colonies per site). The sampled colony on each pallet was rotated on each sample date. Each of these sampled colonies was first assessed at the colony level (Chapter 3) to ensure that there were no queen issues, major parasite or disease presence, or other obvious abnormalities. In this way, the measures gathered were representative of apparently "normal", healthy colonies at each site. As honey bee physiology changes with adult age, it was critical to analyze cohorts of bees at the same approximate ontological time point. To accomplish this goal, newly and recently-eclosing adult bees (≤24 hrs. after eclosion) were marked with a daub of paint (Testor's enamel paint markers, Rockford, IL) on the dorsal surface of the thorax. Care was taken to avoid denting the thorax as the cuticle is not fully hardened in

young teneral adults of this age. Approximately 75 bees were marked per colony. The same paint color was never used twice within a site to eliminate the collection of drifting bees among colonies. Bees were then allowed to develop normally in their colonies of origin for seven days. After seven days, 15 marked bees were recovered from each colony and placed in queen cages provisioned with candy and sprayed with water. The cages with 7-day-old nurse bees were driven (from North Dakota) or shipped (from California) back to the University of Minnesota bee research lab in St. Paul, MN.

4.2.2 Hypopharyngeal glands

In the lab, the heads of 10 of the 15 caged nurse bees from each colony and date were separated from the thorax using a razor blade. The paired hypopharyngeal glands in the head were removed and placed in a glass well plate containing 500µL PBS (Lonza, Walkersville, MD). Gland size was then measured with an ocular micrometer at 400x magnification using a Leica DM100 compound microscope (as in Crailsheim and Stolberg, 1989). The widths of the first 5 acini from each end of each gland were measured and averaged to determine the mean acinus diameter for nurse bees from a given colony on a given date. Glands were suspended in 500µL PBS in a 1.5mL microcentrifuge tube and stored at -80°C for later protein extraction.

4.2.3 Abdominal lipids

The abdomens from the same 10 bees used to determine hypopharyngeal gland size were used to determine the proportion of abdominal weight in lipids. Abdomens were separated from thoraces using forceps, followed by the removal of the alimentary tract (crop, midgut, and hindgut). Each eviscerated abdomen was placed into a clean,

sterile 1.5mL microcentrifuge tube and incubated at 70°C for 24 hrs. After 24 hours, the dry weight of each abdomen was recorded. Next, under a fume hood, 250μL of 1:1 chloroform:methanol was pipetted into each tube, and lids were quickly closed. The dried abdomens were allowed to soak in the chloroform:methanol for 24 hours, after which the chloroform:methanol was removed via vacuum pump. Tubes were then placed back in the drying oven for 24 hours at 70°C. Finally, abdomens were re-weighed and the proportion of weight lost was determined as the percent composition of lipids in the abdomen.

4.2.4 Gene transcript analysis

4.2.4.1 RNA extraction

RNA was extracted from the remaining five 7-day-old bee abdomens from each colony in September of each year only according to the procedure provided with TRIzol® Reagent (Life Technologies, Carlsbad, CA). Briefly, each abdomen was homogenized in 1mL TRIzol in a 1.5mL microcentrifuge tube using a sterile, RNAse, DNAse, and pyrogen free pestle (Kimble Chase, USA). Samples were then centrifuged at 12,000 x g for 10 min. at 4°C. Phase separation was accomplished by collecting the resulting supernatant into a new sterile tube and incubating at room temperature for 5 minutes. Next, 200 μL chloroform (Sigma-Aldrich) was added, and tubes were shaken for 15 seconds. Samples were then incubated again at room temperature for 3 minutes. Tubes were then centrifuged at 12,000 x g for 15 min. at 4°C and the clear supernatant was collected and pipetted into a final sterile 1.5 mL microcentrifuge tube. RNA precipitation was initiated by pipetting 500 μL of 100% isopropanol (Fisher Scientific) into each tube,

inverting tubes 10 times to allow for adequate mixing, and then incubating at room temperature for 10 min. After incubation, samples were centrifuged at 12,000 x g for 10 min. The resulting supernatant was removed, leaving only the RNA pellet at the bottom of the tube. RNA was washed by adding 1 mL 75% ethanol (Decon Labs, Inc.) to each tube and vortexing for 15 seconds, followed by centrifugation at 7500 x g for 5 min. at 4°C. Supernatants were, again, removed and samples were allowed to air dry (on their sides with lids open) for 10 min. at room temperature. RNA was re-suspended by adding 50 µL RNase-free water to each dried pellet and passing the solution up and down through the pipette tip several times (until soluble). Finally, samples were incubated in a water bath at 55-60°C for 15 min. RNA was stored at -80°C for later use in the DNase I and cDNA synthesis steps.

4.2.4.2 qRT-PCR gene transcript expression

Gene mRNA transcript expression levels, relative to ß-Actin, were determined for seven genes in September related to honey bee nutrition and immune function: vitellogenin, insulin-like peptide 1, prophenoloxidase, lysozyme 2, abaecin, defensin 1, and hymenoptaecin. DNA was degraded in the presence of the extracted RNA by preparing a master mix containing 240 U DNase I, 120 µL of 10x DNase buffer, 960 U RNase out, 24 mmol dNTPs, 240 mmol poly$(dT)_{18}$, and 120 µg poly$(dT)_{12\text{-}18}$. An aliquot of 3.1 µL of this master mix was added to each well in a 96-well plate, followed by 8 µL total RNA. A Biorad MyCycler™ thermocycler was then run at 37°C for 1 hour followed by 75°C for 10 min. Next, first-strand cDNA was synthesized by combining 3.9 µL of a superscript master mix containing 100 U Superscript II reverse transcriptase

(Invitrogen), 200 µL of 0.1 M DTT, and 150 µL 5X first-strand buffer to each well and running the thermocycler at 42°C for 50 min. followed by 15 min. at 70°C.

Two µL of cDNA from the previous step were pipetted into each well of a new low-well plate and served as the template to determine the expression of gene transcripts via qPCR. Additionally, 18 µL dH_2O, 0.15 µL of 5U/µL Taq polymerase, 2.5 µL 10x buffer, 0.2 µL dNTPs, 1.5 µL $MgCl_2$, 0.05 µL 10x SYBR Green (Applied Biosystems), and 0.5 µL each of forward and reverse primers for vitellogenin, insulin-like peptide 1, prophenoloxidase, lysozyme 2, abaecin, defensin 1, and hymenoptaecin were added to each well for a total reaction volume of 25 µL (Table 4.1 for primer sequences). Real-time PCR reactions were run in a Biorad C1000™ Thermal Cycler using a thermal profile consisting of 95°C for 10 min., then 94°C for 20 seconds, followed by 40 cycles of a protocol consisting of 4 steps: 95°C for 20 seconds, 60°C for 30 seconds, 72°C for 1 minute, and 78°C for 20 seconds. Fluorescence measures were taken repeatedly at the 78°C step. The 40^{th} cycle was followed by a three-step melt-curve dissociation analysis to confirm amplification of the targeted gene of interest. Expression of each gene of interest was determined as the Cq level (number of cycles for exponential amplification) of ß-Actin – the Cq level of each gene of interest.

4.2.5 Statistical Analysis

Repeated measures analyses of variance were conducted for each individual bee measure across all dates to determine at which time points (if any) a given predictor variable significantly from site to site. Significant differences among factors at the $\alpha \leq 0.05$ level were further examined using the conservative Tukey's multiple comparison

test to determine on which dates variables actually differed. This ANOVA, in addition to investigator knowledge coupled with biological relevance, acted as starting points to inform selection of particular variables and sample dates to include in statistical modeling, and are depicted in Table A1.2, but are discussed only for measures that were significant. No colonies sampled actually died over the winter because, as discussed above, the colonies that were specifically chosen were assessed and determined to be healthy and "normal", and representative of all colonies at each site. Therefore, survival was not in terms of the colonies sampled, but rather apiary survival (as has been analyzed in Chapters 2 and 3). Parameters deemed biologically relevant to the question of *which individual bee measures are useful in predicting colony overwintering survival* were included in statistical modeling using R version 3.1.1 (2014-07-10) using the lme4 package.

The model was created with the response variable: arc-sine square root-transformed annual proportion of colonies surviving, and the factors site and year were included as random effects. All other individual bee predictors were considered as fixed effects. The initial model contained all of the following factors: September expression of vitellogenin (Vg), insulin-like peptide-1 (ilp-1), prophenoloxidase (ppo), lysozyme-2 (lys2), abaecin, defensin-1 (def1), and hymenoptaecin (hym), and August and September hypopharyngeal gland size and abdominal lipids. Backward selection from this large model was employed wherein after running each model, the least significant factor was removed and then the model was re-run. This procedure was carried out until all remaining factors in the model were significant ($p \leq 0.05$). As in the previous chapters, AIC was used to select and confirm that the best of the available models was chosen.

4.3 Results

4.3.1 Statistical model of individual bee measures on annual apiary survival

Three measures from 7-day-old nurse bees were significant predictors of annual apiary survival: 1) nurse bee vitellogenin levels in September, 2) lysozyme-2 expression in September and 3) the relative abdominal lipid mass in August. High levels of Vg and lipids corresponded to greater annual apiary survival, and lower levels of lysozyme-2 were associated with decreased annual apiary survival (as evidenced by the +/- sign of values in Table 4.2). Annual apiary survival (May-March of each year) and mean annual honey production (determined in September of each year) are shown in Table 2.5 for reference.

4.3.1.1 Vitellogenin

Levels of Vg, and all subsequent genes, were analyzed only in 7-day-old adult bees only in September of each year as an indicator of end of season health and predictability on overwinter survivorship. Vitellogenin expression varied significantly across sites ($F_{5,100}$=4.26, p=0.001) and years ($F_{2,100}$=30.4, p=4.9x10^{-11}) (Figure 4.1a). Bees in site 6 exhibited significantly greater levels of Vg compared to bees in sites 2 and 3. In 2012 bees had lower Vg across all sites compared to the other years (Figure 4.1a). Colonies at site 6 had the highest expression levels of Vg and also experienced the greatest survival in all years and were surrounded by the most potential bee forage (Chapter 2). Further, site 6 was one of the sites with the most incoming fresh pollen per day and highest pollen (genera) diversity (Chapter 3).

4.3.1.2 Abdominal lipids

August lipid levels were predictive of annual apiary survival (Table 4.2). Tukey multiple comparison test revealed that significant differences occurred in August lipids between sites 2 and 5, and August lipids did not vary by year (Figure 4.1b). Overall, for all abdominal lipid data, yearly average abdominal lipid levels were highest in 2010 and lowest in 2012 (Figure 4.2). Lipid levels remained relatively constant over time, but were typically greatest in July (concurrently with greatest hypopharyngeal gland size and brood area) and January of each year. The lowest lipid levels generally occurred in August or September (Figure 4.2). Interactions between site and date were significant ($F_{75,474}$=2.15, p=7.5x10^{-7}, Table A1.2), meaning the site with the greatest lipids varied by sample date.

4.3.1.3 Lysozyme-2

Higher September lys-2 levels were associated with lower annual survival (Table 4.2). Each of the three years of the study were statistically distinct with respect to lys-2 levels, with 2011 having the highest levels and 2012 having the lowest (Figure 4.1c). Both site and year were significant for lysozyme-2 gene expression ($F_{5,100}$=2.81, p=0.021; $F_{2,100}$=182.4, p=2x10^{-16}, respectively, with no interaction effects) (Figure 4.1c). Bees in colonies from site 3 exhibited the highest lys-2 immune expression, but were significantly higher only compared to bees from site 2 (Figure 4.1c).

4.3.2 Other individual bee measures

Although the other individual measures, hypophyaryngeal gland size, and gene expression of different components of the immune system, did not significantly predict annual apiary survival in the statistical model, ANOVA of many of the measures by site and date did show interesting significant relationships.

4.3.2.1 Hypopharyngeal gland size

Overall, with respect to year, gland sizes were largest in 2010 and smallest in 2012 (Figure 4.2). Peak hypopharyngeal gland size occurred in July, concurrently with peak lipid levels and brood area (Chapter 3), and again in March (Figure 4.2) when the bees were positioned in CA almond orchards and colonies were beginning to increase in population size and brood rearing. Minimum average size of glands occurred in January, a time when there was very little brood requiring food from the glands (Figure 4.2). Site and date interacted significantly to impact hypopharyngeal gland size ($F_{64,413}$=2.34, $p=2.9x10^{-7}$, Table A1.2), meaning gland size varied by site depending on sample date.

4.3.2.2 Gene expression

The expression of insulin-like peptide-1 was very similar to that of Vg, with significant variation occurring by site ($F_{5,99}$=4.4, p=0.001) and year ($F_{2,99}$=260.63, $p=2x10^{-16}$) (Figure 4.3). Site 6 experienced the greatest expression, while sites 2 and 3 expressed the least ilp-1 and the first two years were characterized by higher ilp-1 expression compared to 2012.

In terms of immune gene expression, prophenoloxidase was determined to vary significantly by site ($F_{5,99}$=3.35, p=0.008) and year ($F_{2,99}$=655.6, p=2x10^{-16}) with no site by year interaction occurring (p>0.05) (Figure 4.4a, Table A1.2). Only sites 3 and 4 were found to significantly differ for ppo expression (higher expression at site 4, Figure 4.4a), an interesting finding considering the similarities in land use at those two sites (Chapter 2). In 2012, ppo levels were lower compared to levels in 2010 and 2011.

Analysis of variance of abaecin expression levels indicated significance by site ($F_{2,100}$=5.07, p<0.000) and year ($F_{2,100}$=10.72, p=6.1x10^{-5}) with no interactions between those main effects (Figure 4.4b). Site three experienced the highest abaecin expression (Figure 4.4b), which was surrounded by the least potential bee forage of any site (Chapter 2), and was one of the sites with the least average incoming fresh pollen per day in 2010 and 2011 (Chapter 3). Here again, significantly lower levels of abaecin were observed in 2012 across all sites compared to 2010-2011 (Figure 4.4b).

Interactions between site and year were detected for defensin-1 ($F_{10,90}$=2.73, p=0.006) and, similar to the expression of abaecin, nurse bees from site three experienced the greatest expression of def-1 (Figure 4.5a, Table A1.2), particularly in years 2010 and 2011 when the greatest differences in survival by site were detected (Chapter 2). Unlike previous genes, defensin-1 in years 2010 and 2012 were similar across all sites, and lower compared to 2011 (Figure 4.5a).

Hymenoptaecin followed a similar pattern to def-1, with a significant interaction occurring between site and year ($F_{10,90}$=5.39, p=3.3x10^{-6}) (Figure 4.5b, Table A1.2). Here again, bees from site 3 experienced the greatest expression of hym (Figure 4.5b), particularly in years 2010 and 2011 when the greatest differences in survival by site were

also detected (Chapter 2). The first two years were statistically similar in expression levels of hym across all sites, while lower levels were detected in 2012.

4.4 Discussion

Three measures of nutrition and immunity in 7-day-old nurse bees were significantly related to annual apiary survival: 1) fall (September) expression levels of vitellogenin (higher levels positively influence survival), 2) lysozyme-2 (higher levels negatively influence survival), and 3) August abdominal lipid levels (higher levels positively influence survival). These measures make sense in light of previous laboratory studies on physiological responses of honey bees to varying nutrition (e.g. Alaux et al., 2010a; Alaux et al., 2011; Di Pasquale et al., 2013) in which both vitellogenin expression and lipid stores were found to increase significantly when bees were fed varying single source and/or mixed pollens. Further, lipid levels are lowest in late summer (Figure 4.2), so having relatively higher levels of lipids in August indicates better nutrition at a time of decreased stores.

While honey bee lys-2 expression has not been well-studied in response to nutrition, it has been shown to be up-regulated in bees responding to certain honey bee-associated bacteria and fungi (Evans, 2006). Expression of lys-2 was similar to that of the three other AMPs examined in our study (abaecin, defensin-1, and hymenoptaecin) in that expression levels were generally highest at the site with the least potential bee forage within a 3.2-km radius (site 3), and lowest at the most bee-friendly forage site (site 6), at least in 2010 and 2011.

Importantly, the colonies chosen for the individual bee measures to represent each site were apparently normal and healthy upon inspection, and none died over any of the

winters. As such, the above measures included in the model represented the background levels of nutrition and immunity in colonies in each of the six sites. This study therefore uncovered physiological indicators of health in individual bees that predicted annual apiary survival in relation to the overall quality of the landscape surrounding the apiary. Even greater variation in measures might have bee observed had all colonies been included in the sampling, including those with queen problems, higher parasite and disease prevalence, and a lack of nutritional stores. Future studies are planned that will measure the individual fates of a broader sample of colonies per apiary over the winter in relation to their fall physiological measures.

Taken together, the results indicate that individual honey bees belonging to colonies positioned in better landscapes (in terms of potential forage in the surrounding lands) possess quantifiably better nutritional status by the end of the foraging season (August and September). Likely as a result of this higher quality nutritional state, bees in those colonies displayed a less activated immune system, as evidenced by their decreased humoral (AMP) immune response. The cellular immune response (total hemocyte counts) was potentially less activated in those sites as well, though data was collected on this measure only in 2012 (data not shown). Conversely, colonies positioned at site 3 with the least area in potential bee forage expressed some of the lowest nutritional stores (Vg and ilp-1) and highest levels of humoral immunity (AMPs). The improved nutritional and immunological state at good quality forage sites sets the stage for more successful overwintering and survival to almond pollination, as evidenced by the differences observed in overwintering survival across the study sites.

One question that remains is how floral resources are allocated by colonies to improve their physiological health and survival. Site 6 had more total area in bee forage. Additionally, and specific land use categories such as CRP were richer in flowers at site 6 compared to site 3, despite a similar total area of CRP in the surrounding landscapes at each apiary site. More pollen per day was brought into colonies at site 6, at least in the first 2 years of the study when survival was significantly different. Despite the abundance of forage and realized amount (mean grams per day) of pollen collected, these resources apparently did not translate into observable differences in colony population size or amount of pollen stored in the colonies by fall (Chapter 3), suggesting that the increased flower abundance and diversity available at site 6 likely was directly consumed by the bees rather than being allocated into colony storage. These findings further highlight the difficulty beekeepers are faced with in obtaining an assessment of colony health that predicts productivity and probability of survival.

The greater overall pollen collection at certain sites with no corresponding change in fall pollen stores may be a reflection of honey bees' storage of relatively constant amounts of pollen and their tight regulation of pollen foraging effort (Seeley, 1995). For example, at sites 5 and 6, colony pollen stores remained relatively constant and no measurable increases in bee population were observed relative to other sites despite the increased pollen foraging effort at site 5 in 2010 and at site 6 in 2010 and 2011. Thus, those protein resources must have been converted into the existing bee population rather than stored, which was manifested in our measurements of the improved nutritional status of bees at those sites.

Another colony manifestation of the increased amount of bee forage at particular sites may have been a potential decreased expenditure of individual bee energy (nutritional) stores and colony-level energy stores (nectar and honey) while locating and collecting pollen and nectar resources in the environment. Colonies experiencing a decreased nutritional status (lower Vg, ilp-1, and lipids) tended to originate from sites with less potential forage, particularly pollen. The colonies at these sites also produced less honey on average.

Pollen diversity varied by site (Chapter 3) with colonies from site 6 collecting the most diverse pollen at the generic level, while colonies from sites 3 and 4 collected the most diverse pollen by family (site 6 was 4th most diverse by family). These differences, as discussed in Chapter 3, may have been due to the relative patch sizes of flowers across the sites, with smaller patches occurring at sites 3 and 4 within many of the uncultivated flower-containing types of land use. As a result, the bees positioned in sites with smaller floral area, patches, and/or more widely-distributed flower resources must have had to search for a more diverse array of flowering plants overall to meet colony demand for protein. Meanwhile, dominant types of land around site 6 (pasture, CRP, hayland) contained relatively large patches of flowers commonly used by bees, but also contained relatively abundant resources within widely distributed areas such as ditches, resulting in ample pollen collection as well as high diversity.

Collecting a diversity of pollen resources might protect against nutritionally deficient (deficiency in amino acids, vitamins, minerals) pollen sources. Collecting pollen from diverse sources may result in colonies having to expend more energy finding and exploiting resources that are sparsely located, and may even bring bees and colonies

into contact with a greater diversity of pesticides. Colonies at sites 4 and 6, for example, collected pollen containing the greatest total number of pesticides (19 and 16 different compounds, respectively) and had the highest pollen hazard quotients (Chapter 3), though PHQ at site six was inflated by a single detection of deltamethrin. At the same time, site 3 pollen contained the fewest pesticides (12) and lowest pollen hazard quotient which was probably due to the low diversity of land use and resulting fewer number of agricultural pesticides utilized at that site.

Gene expression in 2012, particularly for nutritional genes, was significantly lower compared to 2010 and 2011 (Figures 4.1, 4.3, 4.4, and 4.5). These lower measures were in contrast to the increase, or lack of change, in apiary survival in that year (sites 2, 3, 5, and 6). Pollen collection, pollen identification, and honey production were also distinctly different in 2012 (Chapter 2). For example, colonies at site 3 had increased survival and honey production in 2012, but collected less pollen that year compared to the previous two years. The bees may have been utilizing other resources in the surrounding lands outside the 3.2-km radius that was quantified in the study. Therefore gene expression levels should not be thought of in absolute terms but rather are indicative of health and annual survival in a relative sense across all existing sites. If beekeepers were able to obtain measures of gene expression, it would enable them to assess and identify particular sites where colonies are at an increased risk of failure (due to relatively lower nutritional and/or higher immune measures) and thus may need to be closely monitored and possibly treated for parasites and diseases, and/or nutritionally supplemented.

Future research on honey bee colonies positioned across a broader variety of landscapes and beekeeping practices is planned to test how robust the predictors of colony and apiary survival are. Despite the extensive and highly detailed research and observation that has bee carried out on *Apis mellifera* by thousands of scientists and beekeeper across the globe, we still know surprisingly little about the mechanisms involved in processes like nutrient-shunting within an individual bee and among individuals within a colony, and the potential energetic and metabolic trade-offs that may occur between systems such as those involving nutrition and immunity (e.g. DiAngelo et al., 2009). Efforts to identify absolute levels of particular genes and other physiological measures relating to health and survival may prove fruitful in arming beekeepers and researchers alike with a reliable method to accurately and objectively assess the status of their hives. Such a "blood test" for bees would be a breakthrough in the ongoing struggle to maintain healthy, live colonies of honey bees to meet the current demands for pollination, support a robust beekeeping industry, and ensure a safe and reliable food system in an increasingly human-modified world.

This study uncovered physiological measures of honey bee health and immunity relating to apiary-level phenomena, i.e. apiary survival, influenced by landscape suitability. While there is much interest and excitement exist regarding the study of the effects of land use on honey bee (and other pollinator) health and survival, little data exist on which to build coherent real-world policies that will significantly improve the situation for honey bees and other pollinators. As such, this study should be viewed as a novel first step in identifying pertinent physiological responses that honey bees may have

as a result of their positioning near varying landscape factors in intensive agricultural environments.

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4.6 Table legends

Table 4.1 Primer sequences (forward and reverse) and references used for nutritional and immune gene expression analysis.

Table 4.2 Statistical model of individual bee data on annual apiary survival. Apiary survival was arc-sine square root transformed and site and year were included as random effects. All other effects (Vg, lys2, lipids) were analyzed as fixed effects.

4.7 Tables

Table 4.1

Gene name	Direction	Primer Sequence	Reference
β-Actin	forward	TTGTATGCCAACACTGTCCTTT	Simone et al., 2009
	reverse	TGGCGCGATGATCTTAATTT	
Vitellogenin	forward	AGTTCCGACCGACGACG	Corona et al. 2007
	reverse	TTCCCTCCCACGGAGTCC	
Insulin-like Peptide 1	forward	GCTCAGGCTGTGCTCGAAAAGT	Corona et al. 2007
	reverse	CGTTGTATCCACGACCCTTGC	
Pro-phenoloxidase	forward	GTTTGGTCGACGGAAGAAAA	Evans, 2006
	reverse	CCGTCGACTCGAAATCGTAT	
Lysozyme 2	forward	CCAAATTAACAGCGCCAAGT	Evans, 2006
	reverse	GCAATTCTTCACCCAACCAT	
Abaecin	forward	CAGCATTCGCATACGTACCA	Evans, 2006
	reverse	GACCAGGAAACGTTGGAAAC	
Defensin 1	forward	TGCGCTGCTAACTGTCTCAG	Evans, 2006
	reverse	AATGGCACTTAACCGAAACG	
Hymenoptaecin	forward	CTCTTCTGTGCCGTTGCATA	Evans, 2006
	reverse	GCGTCTCCTGTCATTCCATT	

Table 4.2

Model (Apiary Survival)	**Fixed Effect**	**Value**	**SE**	**df**	**T-value**	**P-value**
$\sin^{-1}(\sqrt{(\text{prop. survival})}) \sim$	Intercept	0.74	0.17	12.15	4.3	0.001
Sep. vitellogenin +	Sep. Vg	0.07	0.02	10.02	3.4	0.006*
Aug. lipids +	Aug. Lipids	1.58	0.68	11.78	2.3	0.038*
Sep. lysozyme-2	Sep. Lys-2	-0.05	0.01	5.18	-3.3	0.02*
Random Effects	**Intercept**	**Variance**	**S.D.**			
	Site	0.002	0.04			
	Year	0.002	0.04			
	Residual	0.004	0.06			

4.8 Figure legends

Figure 4.1 Statistically modeled significant individual bee measures, including September vitellogenin (1a), August abdominal lipids (1b) and September lysozyme-2 (1c). Significant differences were determined using ANOVA and Tukey's honest significant difference multiple comparisons test. Box and whisker plots depict the median (black line), upper and lower quartiles (the box, 25% of the data is greater or less, respectively), maximum and minimum (whiskers) not including outliers, and outliers (open circles) greater or less than 1.5 times the upper or lower quartile, respectively.

Figure 4.2 Seasonal hypopharyngeal gland size (mm) and proportional abdominal lipid mass (mg) ± s.e., among the six sites and three years of the study.

Figure 4.3 Insulin-like peptide transcript expression by site (1-6) and year (2010-12). Main effect significant differences by site and year were determined by Tukey HSD multiple comparisons test.

Figure 4.4 Immune gene expression for genes where main effects (site and year) were significant (no interaction between main effects), 2010-12, including prophenoloxidase (4a) and abaecin (4b). Significant differences by site and year were determined by Tukey HSD multiple comparisons test.

Figure 4.5 Immune gene expression for genes with interactions occurring between main effects (site x year), Only main effects are shown. September 2010-12, defensin-1 (5a) and hymenoptaecin (5b). Significant site by year differences were determined by Tukey HSD (only significant differences for sitet and year are shown here).

4.9 Figures

Figure 4.1

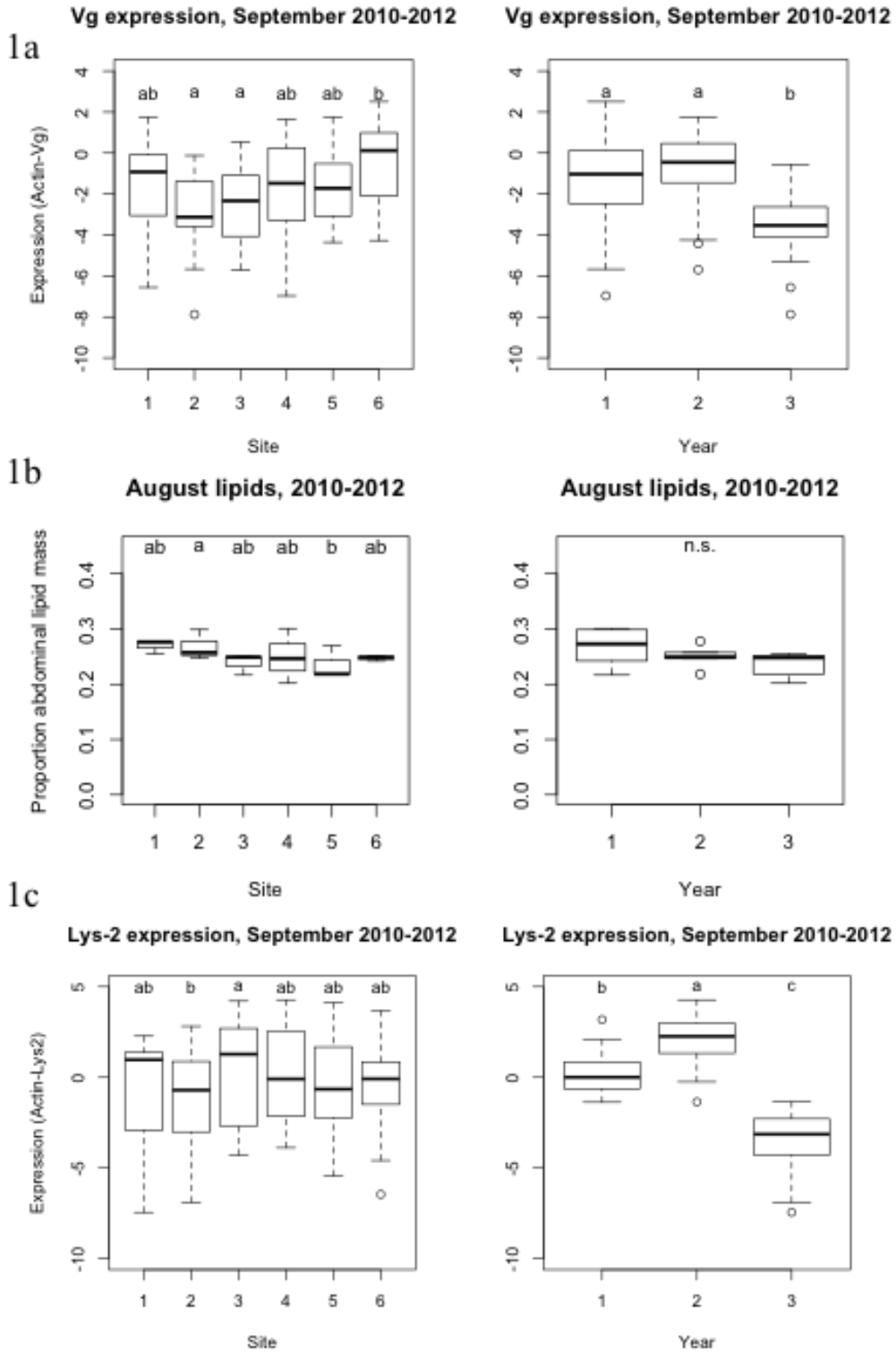


Figure 4.2

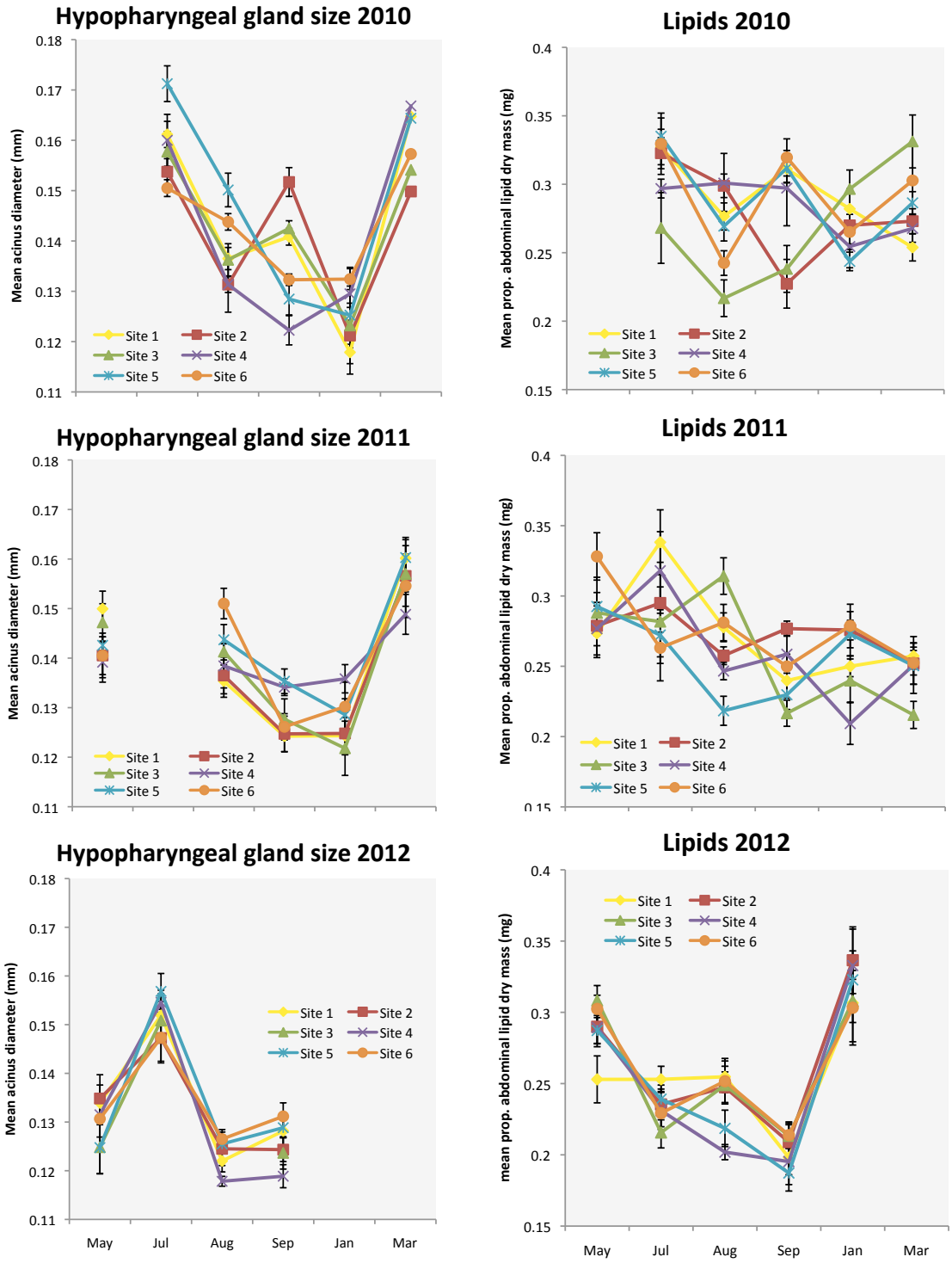

Hypopharyngeal gland size 2010
Mean acinus diameter (mm)
0.18
0.17
0.16
0.15
0.14
0.13
0.12
0.11
Site 1
Site 2
Site 3
Site 4
Site 5
Site 6
Lipids 2010
Mean prop. abdominal lipid dry mass (mg)
0.4
0.35
0.3
0.25
0.2
0.15
Site 1
Site 2
Site 3
Site 4
Site 5
Site 6
Hypopharyngeal gland size 2011
Mean acinus diameter (mm)
0.18
0.17
0.16
0.15
0.14
0.13
0.12
0.11
Site 1
Site 2
Site 3
Site 4
Site 5
Site 6
Lipids 2011
Mean prop. abdominal lipid dry mass (mg)
0.4
0.35
0.3
0.25
0.2
0.15
Site 1
Site 2
Site 3
Site 4
Site 5
Site 6
Hypopharyngeal gland size 2012
Mean acinus diameter (mm)
0.18
0.17
0.16
0.15
0.14
0.13
0.12
0.11
Site 1
Site 2
Site 3
Site 4
Site 5
Site 6
May
Jul
Aug
Sep
Jan
Mar
Lipids 2012
mean prop. abdominal lipid dry mass (mg)
0.4
0.35
0.3
0.25
0.2
0.15
Site 1
Site 2
Site 3
Site 4
Site 5
Site 6
May
Jul
Aug
Sep
Jan
Mar

Figure 4.3

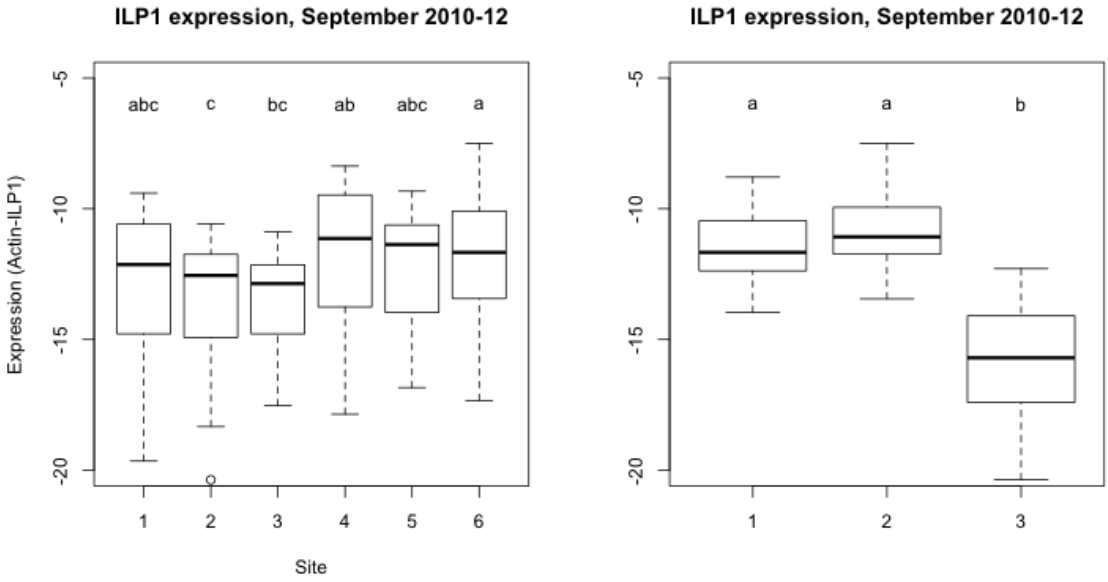
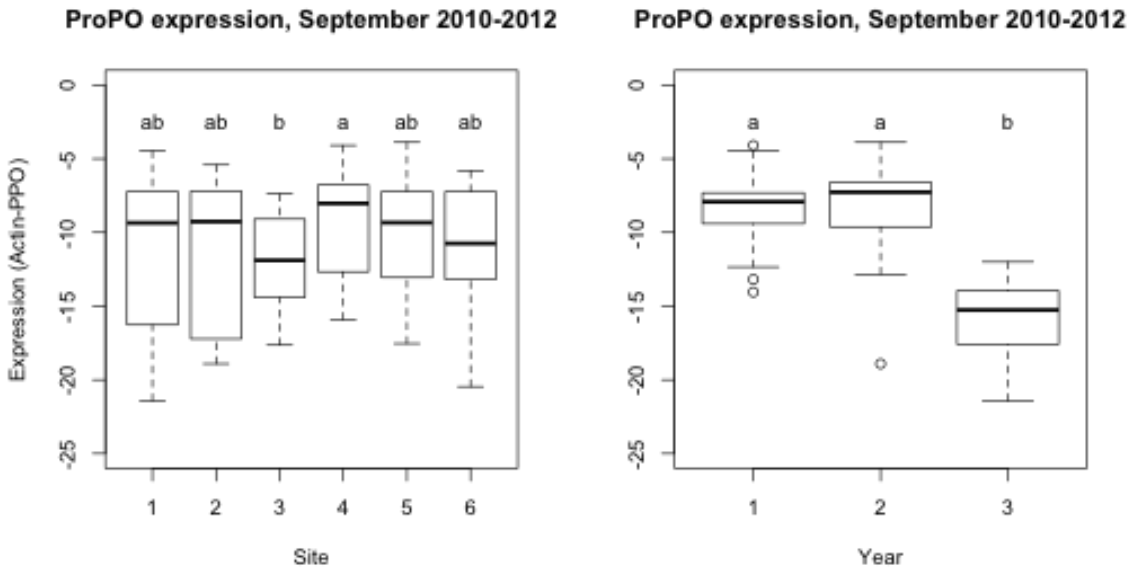

ILP1 expression, September 2010-12
Expression (Actin-ILP1)
-5
-10
-15
-20
abc
c
bc
ab
abc
a
1
2
3
4
5
6
Site
ILP1 expression, September 2010-12
a
a
b
1
2
3

Figure 4.4

4a



4b

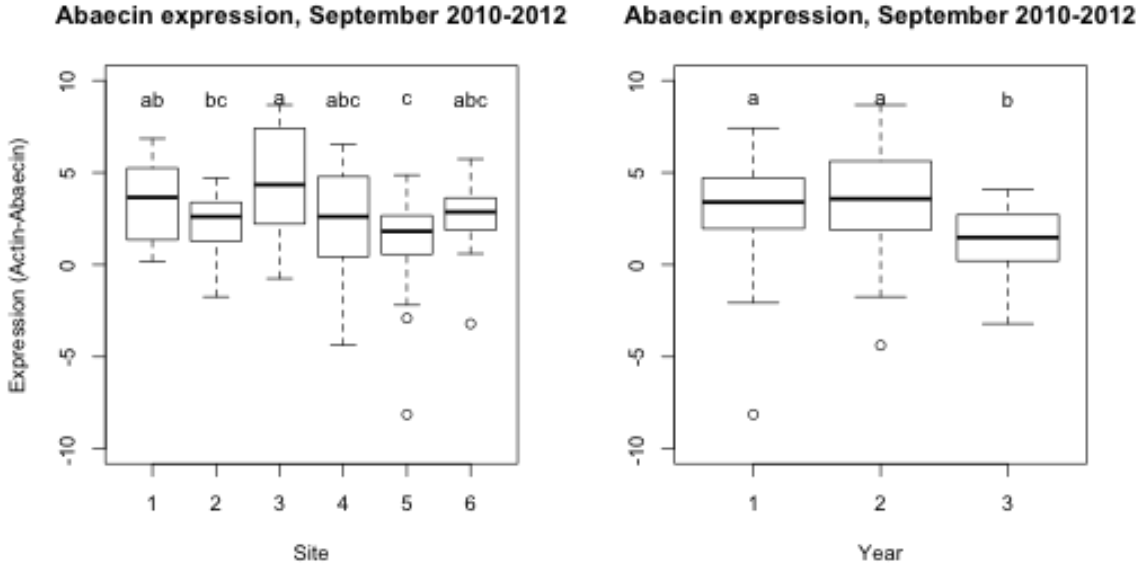
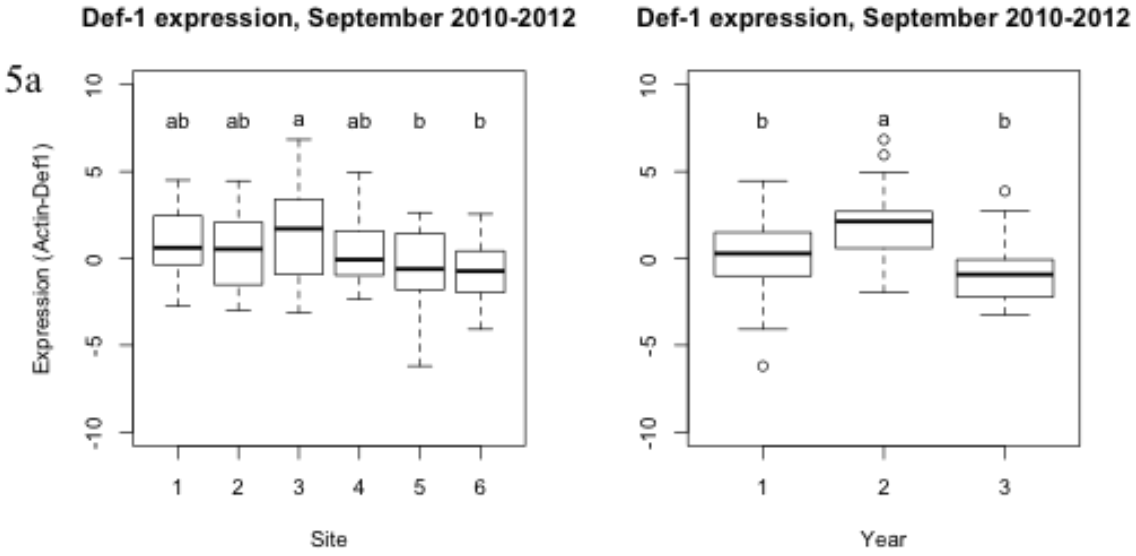
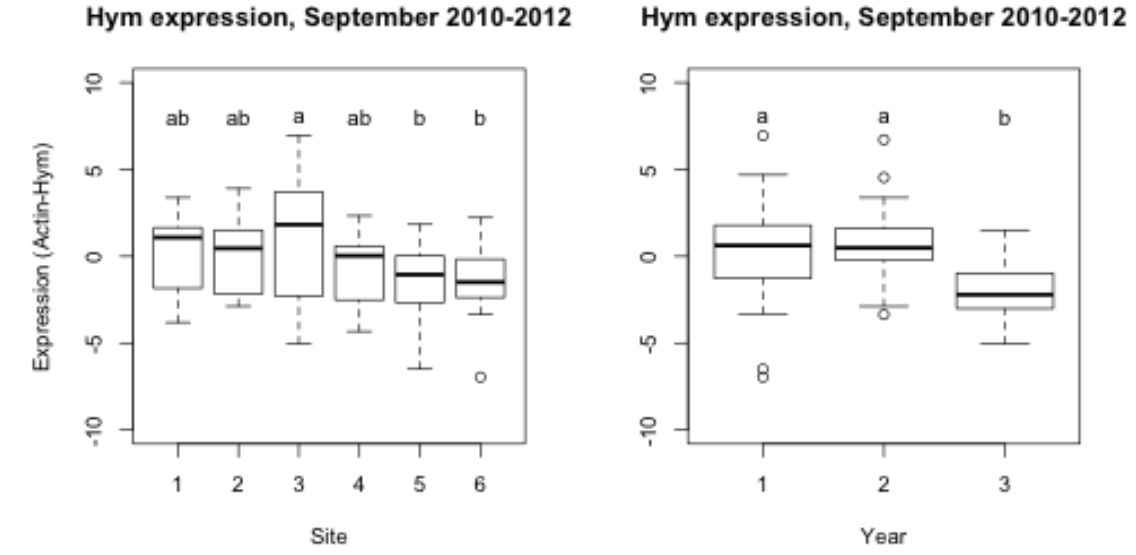


Figure 4.5

5a



5b



CHAPTER 5

Consolidation of land, colony, and individual measures into a single model.

Matthew Smart

5.1 Introduction

The previous chapters explored factors that had measureable impacts on honey bee colony health and survival across three years in a beekeeping operation in the Prairie Pothole Region of North Dakota. The parameters were divided into three levels: 1) land use and the availability of flowering plants in a 3.2-km radius around six apiaries; 2) measures of the health of bee *colonies* over time; and 3) physiological measures of health of *individual bees* within the colonies over time. Here, the parameters that were found to be significant at each level were combined into a final model. The aims were to determine which parameters were most important in predicting overall colony survival, and if any measures were redundant; that is, significantly correlated with each other. The ultimate goal was to gain an understanding of how land use impacts the health and ability of bee colonies to produce honey, meet pollination demands, and ultimately survive in a migratory beekeeping operation.

5.2 Methods

The model parameters from the analysis of landscape (Chapter 2), colony (Chapter 3), and individual bee (Chapter 4) that significantly impacted the proportion of colonies surviving over three years (from May through the following March) were inserted into a new "grand model". This model initially started with the following parameters: the area (log-transformed m^2) of uncultivated bee forage in a 3.2-km radius

around each of the six apiaries during the summer in North Dakota, the number of combs of pupating (sealed) brood in September, the level of *Varroa* mite infestation in the colonies in September (mites per 100 adult bees), the average incoming amount (grams) of fresh pollen collected per colony per day, the expression levels of vitellogenin and lysozyme-2 relative to ß-actin in 7-day-old adult bees in September, and the mean proportion of abdominal stored lipids in 7-day-old adult bees in August. The model was produced using R version 3.1.1 (2014-07-10). The response variable was the annual proportion of colonies surviving (arc-sine square root-transformed) with the factors site and year modeled as random effects. All other parameters were considered as fixed effects. Backward selection from this large model was employed wherein after running each model, the least significant factor was removed and then the model was re-run. This procedure was carried out until all remaining factors in the model were significant ($p \leq 0.05$). Akaike Information Criterion (AIC) was used to select and confirm that the best of the available models was chosen. Residuals were plotted and checked for homoscedacity, and linearity of the data were checked using added-variable plots, and data was confirmed to conform to model assumptions. The final model is depicted in Table 5.1.

5.3 Results

All initial model predictors remained in the final model with the exception of September *Varroa* infestation level (Table 5.1). Five of the parameters that remained in the final model: the amount of bee forage around the apiaries, large sealed brood areas in September, large amounts of incoming pollen in August and September, and high levels

of vitellogenin and lipid in young bees, had a positive influence on colony survival. The sixth parameter, lysozyme-2 expression had a negative influence on colony survival.

The area (m^2) of uncultivated bee forage (including pasture, CRP, grassland, fallow land, flowering trees and shrubs, hayland, and ditch) within the typical forage area of a honey bee colony was influential in supporting honey production, and more importantly, colony survival (Chapter 2). The area of pupating (sealed) brood within the colonies in September, and the average daily amount (grams) of fresh pollen collected by colonies were positively correlated with the area of forage, though not significantly (September brood: $F_{1,16}$=0.2, r^2=0.01. p=0.66; amount of pollen: $F_{1,16}$=2.2, r^2=0.12. p=0.16). Vitellogenin expression and weight of abdominal lipids in 7-day-old nurse bees were also positively correlated, though not significantly, with the area of bee forage (vitellogenin: $F_{1,16}$=1.2, r^2=0.07, p=0.28; August lipids: $F_{1,16}$=0.64, r^2=0.04, p=0.43). The transcript abundance of lysozyme-2 in 7-day-old bees was not correlated or significant (lysozyme-2: $F_{1,16}$=0.04, r^2=0.002, p=0.84). Thus, while land use patterns were related to colony and individual bee parameters, they were not statistically redundant measures. Therefore, all measures, except for September levels of *Varroa* mites, were integral in predicting colony health and survival.

5.4 Discussion

I have shown that six measures, including the availability of forage in the surrounding agricultural landscape, and nutritional and immunological responses of bees to the landscape, significantly influence annual colony survival. The six factors that significantly impact bee survival include landscape, colony-level and individual bee-level parameters that are not mutually exclusive, but are independent predictors of health and

survival. This is the first time such a comprehensive analysis has been conducted and represents a significant step toward a more complete picture of how land use impacts the ability of honey bee colonies to produce honey, meet pollination demands, and ultimately survive within a commercial, migratory beekeeping setting.

5.5 Project conclusions

5.5.1 Landscape level

Overall, apiaries surrounded by land containing the greatest area in uncultivated potential bee forage (e.g. site 6) experienced the greatest honey production and survival over the three years of study (Chapters 2 and 3). Uncultivated forage area, but not honey production, was predictive of annual apiary survival. Over the three years of the experiment, land use surrounding the six sites changed very little in general. The Shannon-Weiner diversity index of land use varied by site, though interestingly revealed that the best site for honey production and survival (site 6) was not the most diverse. The worst performing site for honey production and annual survival, site 3, was surrounded by land containing the least diversity, being dominated by agricultural commodity crops of little value to honey bees. These results suggest that while a low diversity profile of mostly non-bee forage crops is detrimental to honey bee colonies, in increase in diversity *per se* does not continue to improve outcomes for honey bee colonies past a certain point. Rather, abundance of particular types of land use (e.g. pasture, CRP, grassland) rather than high overall diversity in land use is preferable for honey bee productivity and survival.

Further, land use categories among sites were not all equal in terms of quality. For example, despite similar total areas of CRP surrounding sites 3 and 6, floral cover estimates were vastly different; approximately 24% and 74%, respectively. Therefore, the land in uncultivated forage, as well as the overall "quality" of those lands (availability of floral resources for honey bees), were important for colony health and survivorship. Survivorship analyses showed that most colony losses among all six sites primarily occurred over the winter, and in the first two years of the study, site 3 experienced significantly greater losses compared to the other five sites.

5.5.2 Colony level

At the colony level, three measures were predictive of annual apiary survival, 1) the fresh weight (g) of pollen collected per day, 2) the (pupal) brood population size in September, and 3) the *Varroa* mite infestation rate (mites per 100 adult bees) in September (Chapter 3). Greater annual survival was observed and predicted in apiaries in which colonies had higher pollen collection levels and September brood population sizes. As predicted, higher *Varroa* infestation rates were associated with decreased annual apiary survival. While higher brood levels in September were related to better annual survival, overall adult and immature population sizes were not statistically different among sites. Similarly, pollen stores in the fall were not significantly different among sites. In fact, while site 6 had some of the greatest amounts of stored pollen (Figure 3.3) so did site 3, suggesting that colony pollen stores do not fluctuate to a large degree. Interestingly, sites 1 and 2, the most diverse in terms of land use, had relatively low pollen stores in 2010 and 2011.

Pollen collection (g/day) was a significant predictors of apiary survival, and there was a positive relationship between pollen collection and uncultivated forage lands. Pollen collection varied by site and date, and due to the high degree of observed variance (Figure 3.4), statistical significance was not detected among sites. Given the fact that overall bee population sizes and pollen stores were not statistically different among sites, the "extra" pollen collected at sites 5 and 6 (for example in 2010 and 2011) must have been directly consumed by the bees in those colonies at the time of the pollen's return. These additional resources, above those of colonies located at other sites, apparently improved the overall nutritional status of the bees in those colonies as measured by the greater physiological health of the bees in colonies at those sites (discussed in Chapter 4 and below).

Overall, 18 families and 33 genera of flowering plants were detected among the six sites and three years of study. Three families: Asteraceae, Brassicaceae, and Fabaceae made up the majority of bee-collected pollen in these landscapes, comprising up to 57%, 26%, and 81%, respectively of the total pollen among all sites and years. Of particular note within the Fabaceae was the genus *Melilotus* spp. (white and yellow sweet clover) that alone made up 18-34% of the total pollen among all sites over all three years. In contrast, cultivated plants made up relatively little of the pollen foraged by honey bee colonies in these lands, just 3-17% among all sites and years. Other plants growing in uncultivated forage areas of importance included Asteraceae: *Sonchus* spp., *Grindelia squarrosa*, *Solidago* spp., *Circium* spp., and *Cichorium* spp.; Fabaceae: *Melilotus* spp., *Medicago sativa*, *Trifolium* spp., and *Vicia*. The Shannon-Weiner diversity index of pollen (family and generic level) indicated that, in contrast to the land use diversity index,

site 3 collected the most diverse pollen. Sites 1 and 2 conversely collected the least diverse pollen. These results, taken together with the land use areas in uncultivated forage and the overall floral cover estimates within each type of land use suggest that honey bee colonies positioned in sites of low land diversity and percent floral cover overall may exhaust the relatively few large patches of attractive flowers and then be forced to move on to small patches and/or more widely distributed resources in their surroundings. The result is that those colonies may come into contact with a greater overall diversity of flowering plants but at the potential detriment to colony productivity. Further, if resources are more patchy and/or widely dispersed, one would expect to observe an effect on the overall amount of resources collected by those colonies. This, in fact, was manifest in our measures of honey production as well as pollen collection, at least when comparing site 3 to site 6.

At the outset of the study pesticide exposure was predicted to be highest among sites with greater agricultural land use nearby, and lowest among sites with the least area of surrounding lands in intensively managed agricultural crops. This prediction was ultimately partially supported and partially refuted. While low pesticide exposure profiles occurred at sites 2 (located within a wildlife refuge) and 6 (when the single detection of deltamethrin is removed) as predicted, the lowest overall PHQ occurred at site 3 which was surrounded by the most area in intensive agricultural crops. The observed pesticide residue profile results are likely related to farmers' use of pesticides in the lands around the sites and the timing of pollen collection for pesticide residue analysis. As pesticide applications were not coordinated with the beekeeper, pollen samples may have occurred at any time before, during, or after sprays. Therefore, care

should be taken in making definitive conclusions regarding pesticide exposure in relation to colony outcomes from the present data set.

5.5.3 Individual bees level

Three measures of nutrition and immunity in 7-day old nurse bees were predictive of annual survival, 1) the expression of vitellogenin in September, 2) abdominal lipid stores in August, and 3) the expression of lysozyme-2 in September. The first two measures (vitellogenin and lipids) were positively correlated with survival, while higher lysozyme-2 expression levels were associated with decreased annual survival. All three measures have been previously shown to be affected by nutritional status and/or immune challenge, and therefore highlight the interactions occurring between those two systems and the resulting outcomes for colonies (survival and productivity). The relationship between greater nutritional stores and lower immune system activation in apiaries experiencing greater annual survival was corroborated by the expression of the other nutritional gene measured, insulin-like peptide-1 (significantly higher at site 6) , and the three anti-microbial peptide genes analyzed: abaecin, defensin-1, and hymenoptaecin (significantly lower at sites 5 and 6).

5.5.4 Overall conclusions and future studies

The most significant predictors of health and survivorship across all three levels of analysis were all related to nutrition - beginning with abundant flowers located overwhelmingly in uncultivated lands. More and/or better forage led to greater honey production and pollen collection, which in turn led to greater nutritional stores in individual bees, and an overall decreased immune response. The presence of quality and abundant forage surrounding summering locations support healthy, robust, and most

importantly, surviving, colonies of honey bees. The flowers the honey bee colonies relied upon in this region were primarily from lands that were unmanaged (for honey bees) and included plants that may be non-native and considered "weedy" by some. However, given the extreme importance of the region for summering managed honey bees colonies (40-50% of all commercially pollinating hives summer in the upper Midwest of the U.S.), I would call for a rational and open-minded discussion examining current policies and management practices as they pertain to certain plants on which honey bees particular rely (i.e. *Melilotus* spp.). At the same time I would emphasize the need for further research into potential alternative, less controversial, species that would benefit and support honey bee colonies at their current densities across the region.

Beekeepers, equipped with this knowledge, now have the ability to 1) make better site selection decisions by identifying specific types of land use (pasture, CRP, grassland, fallow land, flowering woody plants, hayland, and ditch) likely contributing to better honey production and survival outcomes, 2) utilize a more robust suite of measurements to assess hive health and intervene early if possible, when management inputs are most likely to result in improved outcomes for their colonies, and 3) select breeder queens from colonies that have established themselves to possess a suite of measures indicative of overall good health.

There is a complex interplay between colony nutrition, parasite and disease presence, pesticide exposure, and resulting physiological responses of individual bees, leading to survival or mortality of colonies. The nuanced interactions among these factors are difficult to disentangle in field studies. However, colony nutrition, specifically the amount and diversity of protein (pollen) they have access to, was a

critical factor linking colony growth and bee health generally, and specifically the significant parameters in this study (brood area, Vg, lipids).

Future studies are needed to determine if the results of this grand model can be generalized to other regions and beekeeping management styles. For example, the greatest challenge most beekeepers face is the effective control of *Varroa* mite populations. Clearly, the participating beekeeper was able to maintain mite population levels below colony and economic injury levels (Chapter 3), so negative effects of *Varroa* on colony health were not apparent. However, this may not be the case for other beekeepers using different management styles or in other regions of the country that do not keep mite levels under 3-5 mites per 100 bees (due to interactions between climate, pesticide efficacy, and varying strains of mites). Likely there will be regional variations, but the parameter of available bee forage in uncultivated lands should be relatively robust provided that alternative forage by region are similar in quality (for honey bees) to what we have observed here. That being said, regional forage options for growers and landowners should be better studied and incorporated into programs such as CRP and potentially as cover crops and in field margins.

An additional future question would be whether individual colonies that gather more, or more diverse, pollen resources due to a greater efficiency in recruiting and/or foraging do better even in relatively poor landscapes. Colonies that behave as such may be more adept at locating and exploiting the sparse and patchy resources indicative of poor sites. Such individual colonies could then be selected as potential breeder colonies (colonies from which to raise queens or collect drone semen for queen insemination).

5.6 Table legend

Table 5.1 Statistical model of landscape, colony, and individual bee data on annual apiary survival. Apiary survival was arc-sine square root transformed and site and year were included as random effects. All other effects were analyzed as fixed, including area (m2) uncultivated and un-mowed forage, combs of pupating brood in September, mean fresh weight (g) of incoming pollen per 24-hr period, September vitellogenin expression in 7-day-old adult bees, September lysozyme-2 expression in 7-day-old adult bees, and August abdominal lipid mass in 7-day-old adult bees.

5.7 Table

Table 5.1

Model (Apiary survival)	Effect	Value	SE	DF	T-value	P-value
$\sin^{-1}(\sqrt{(\text{prop. survival})}) \sim$ log(area uncultivated forage) + Sep brood + fresh pollen weight/day + Sep Vg + Sep Lys2 + Aug lipids	Intercept	-0.69	0.56	4.78	-1.23	0.28
	Forage	0.09	0.03	4.82	2.55	0.05*
	Sep Brood	0.21	0.05	7.98	3.83	0.005*
	Pollen (g)	0.0007	0.0002	7.39	2.99	0.02*
	Sep Vg	0.03	0.01	8.65	2.52	0.034*
	Sep Lys 2	-0.02	0.008	4.82	-2.74	0.042*
	Aug Lipids	1.01	0.36	7.41	2.76	0.027*
Random Effects	**Intercept**	**Variance**	**SD**			
	Site	0.002	0.049			
	Year	0.0001	0.011			
	Residual	0.001	0.032			

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Appendix 1. ANOVA tables for colony and individual bee analyses, 2010-2013

Table A1.1

Metric/Measure	Effect	DF	Sum Sq	Mean Sq	F-value	P-value
Frames of bees	Site	5	403	80.6	10	$1.7x10^{-9}$
	Date	20	$1.4x10^{4}$	689.3	85.46	$2x10^{-16}$
	Site*Date	100	2080	20.8	2.58	$6.4x10^{-15}$
	Residuals	2383	$2.2x10^{4}$	8.1		
Frames of sealed (pupating) brood	Site	5	24.6	4.92	11.17	$1.1x10^{-10}$
	Date	19	2568.5	135.19	306.72	$2x10^{-16}$
	Site*Date	95	76	0.8	1.82	$3.7x10^{-6}$
	Residuals	2561	1128.8	0.44		
Frames of pollen	Site	5	37.5	7.5	17.11	$4.2x10^{-16}$
	Date	5	79.2	15.84	36.17	$2x10^{-16}$
	Site*Date	25	34.5	1.38	3.15	$4.5x10^{-7}$
	Residuals	801	350.8	0.44		
Fresh weight (g) incoming pollen	Date	13	$2.8x10^{5}$	$2.1x10^{4}$	2.53	0.003
	Site	5	$2.1x10^{5}$	$4.3x10^{4}$	5.12	0.0002
	Date*Site	65	$7.8x10^{5}$	$1.2x10^{4}$	1.42	0.038
	Residuals	168	$1.4x10^{6}$	$8.4x10^{3}$		
Honey	Site	5	$1.0x10^{5}$	$2.0x10^{4}$	12.2	$4.8x10^{-11}$
	Date	2	$1.3x10^{5}$	$6.7x10^{4}$	40.3	$2.0x10^{-16}$
	Site*Date	10	$1.2x10^{5}$	$1.2x10^{4}$	7.1	$2.8x10^{-10}$
	Residuals	414	$6.8x10^{5}$	1652		
Varroa mite infestation rate	Site	5	83.7	16.75	16.32	$7.3x10^{-16}$
	Date	17	476.8	28.05	27.34	$2x10^{-16}$
	Site*Date	85	331.5	3.9	3.8	$2x10^{-16}$
	Residuals	2375	2436.9			
Nosema spp.	Site	5	$1.9x10^{14}$	$3.8x10^{13}$	17.41	$2x10^{-16}$
	Date	17	$5x10^{15}$	$2.9x10^{14}$	134.01	$2x10^{-16}$
	Site*Date	85	$6.5x10^{14}$	$7.7x10^{12}$	3.5	$2x10^{-16}$
	Residuals	2374	$5.2x10^{15}$	$2.2x10^{12}$		
ABPV	Site	5	595	119.02	12.09	$6.1x10^{-11}$
	Date	13	1307	100.53	10.21	$2x10^{-16}$
	Site*Date	65	1822	28.03	2.85	$1.6x10^{-10}$
	Residuals	410	4036	9.84		
BQCV	Site	5	310	62	4.6	0.0004
	Date	13	5127	394.4	29.25	$2x10^{-16}$
	Site*Date	65	1704	26.2	1.95	$5.9x10^{-5}$
	Residuals	410	5529	13.5		
CBPV	Site	5	153	30.69	1.33	0.251
	Date	13	2288	176.01	7.62	$8.9x10^{-14}$
	Residuals	475	$1.1x10^{-4}$	23.09		
DWV	Site	5	231	46.2	0.67	0.65
	Date	13	$1.6x10^{4}$	1264.5	18.2	$2x10^{-16}$
	Site*Date	65	8487	130.6	1.88	0.0001
	Residuals	410	$2.8x10^{4}$	69.5		
IAPV	Site	5	1123	224.6	7.72	$5.9x10^{-7}$
	Date	13	8307	639	21.97	$2x10^{-16}$
	Site*Date	65	4008	61.7	2.12	$5.8x10^{-6}$

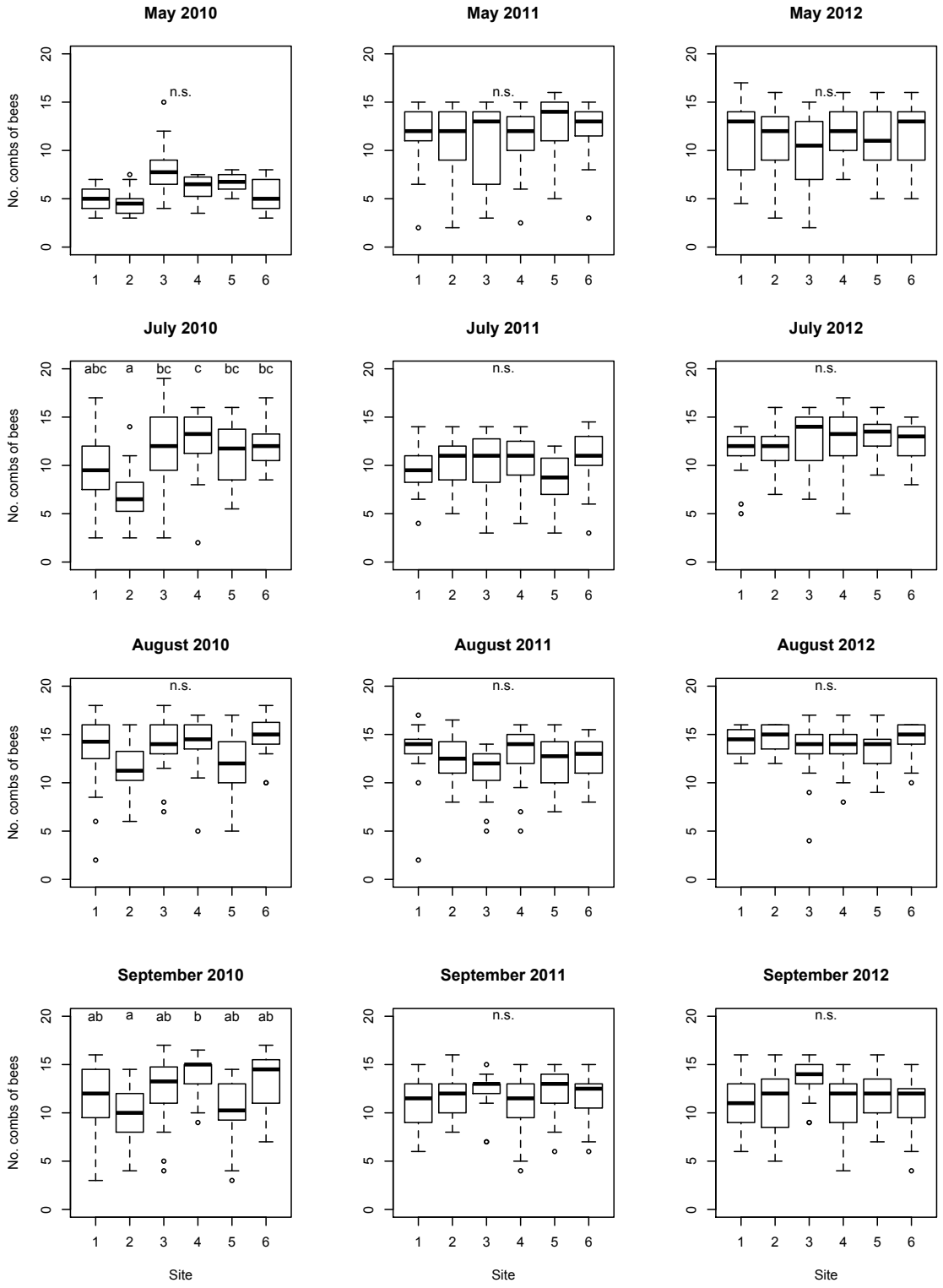
Metric/Measure	Effect	DF	Sum Sq	Mean Sq	F-value	P-value
	Residuals	410	$1.2x10^4$	29.1		
KBV	Site	5	43	8.64	1.09	0.37
	Date	13	1965	151.12	19.04	$2x10^{-16}$
	Residuals	475	3769	7.94		
SBV	Site	5	697	139.5	2.39	0.037
	Date	13	$1.5x10^4$	1182.3	20.29	$2x10^{-16}$
	Site*Date	65	5577	85.8	1.47	0.014
	Residuals	410	$2.4x10^4$	58.3		

Table A1.2

Measure	Effect	DF	SS	MS	F-value	P-value
Hypopharyngeal Gland Size (mm)	Site	5	0.001	0.0002	2.25	0.049
	Date	13	0.079	0.0061	69.15	$2x10^{-16}$
	Site*Date	64	0.013	0.0002	2.34	$2.9x10^{-7}$
	Residuals	413	0.036	0.0001		
Abdominal Lipids	Site	5	0.023	0.005	3.33	0.006
	Date	15	0.46	0.031	22.08	$2x10^{-16}$
	Site*Date	75	0.225	0.003	2.15	$7.5x10^{-7}$
	Residuals	474	0.659	0.001		
Vitellogenin	Site	5	55.96	11.19	4.26	0.001
	Year	2	159.56	79.78	30.4	$4.9x10^{-11}$
	Residuals	100	262.49	2.62		
Insulin-like Peptide 1	Site	5	51.2	10.24	4.40	0.001
	Year	2	521.3	260.63	112.1	$2x10^{-16}$
	Residuals	99	230.1	2.32		
PPOact	Site	5	92.5	18.5	3.35	0.008
	Year	2	1311.1	655.6	118.5	$2x10^{-16}$
	Residuals	99	547.5	5.5		
Lysozyme 2	Site	5	22.5	4.51	2.81	0.021
	Year	2	586.2	293.1	182.4	$2x10^{-16}$
	Residuals	100	160.6	1.61		
Abaecin	Site	5	137	27.4	5.07	< 0.000
	Year	2	115.9	57.93	10.72	$6.1x10^{-5}$
	Residuals	100	540.5	5.41		
Defensin 1	Site	5	67.15	13.43	4.66	0.001
	Year	2	148.44	74.22	25.77	$1.4x10^{-9}$
	Site*Year	10	78.56	7.86	2.73	0.006
	Residuals	90	259.22	2.88		
Hymenoptaecin	Site	5	89.06	17.81	5.82	< 0.000
	Year	2	151.8	75.9	24.81	$2.6x10^{-9}$
	Site*Year	10	164.88	16.49	5.39	$3.3x10^{-6}$
	Residuals	90	275.3	3.06		

Appendix 2. Figures of all colony measures by site and date, 2010-2013

Figure A2.1



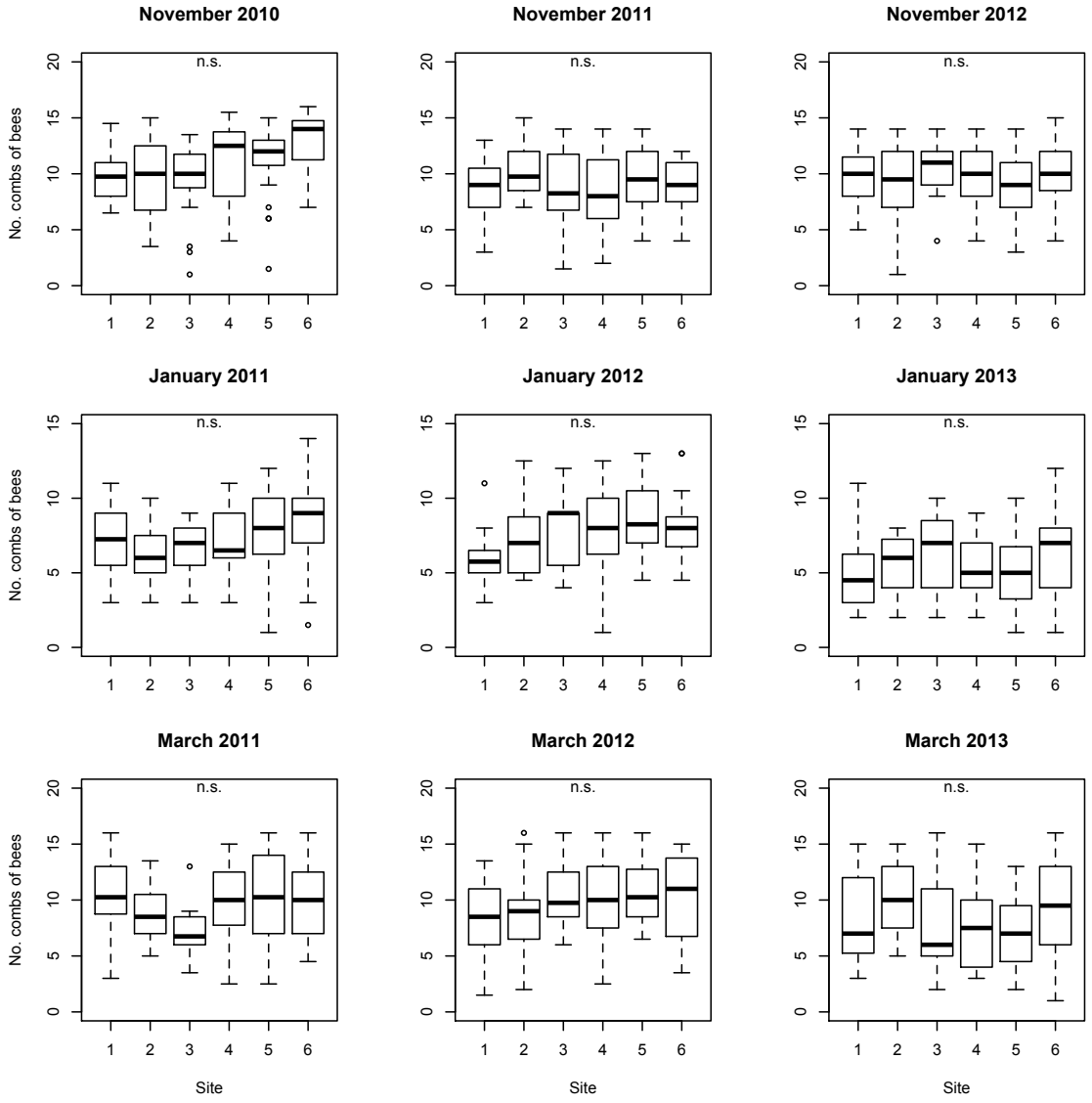
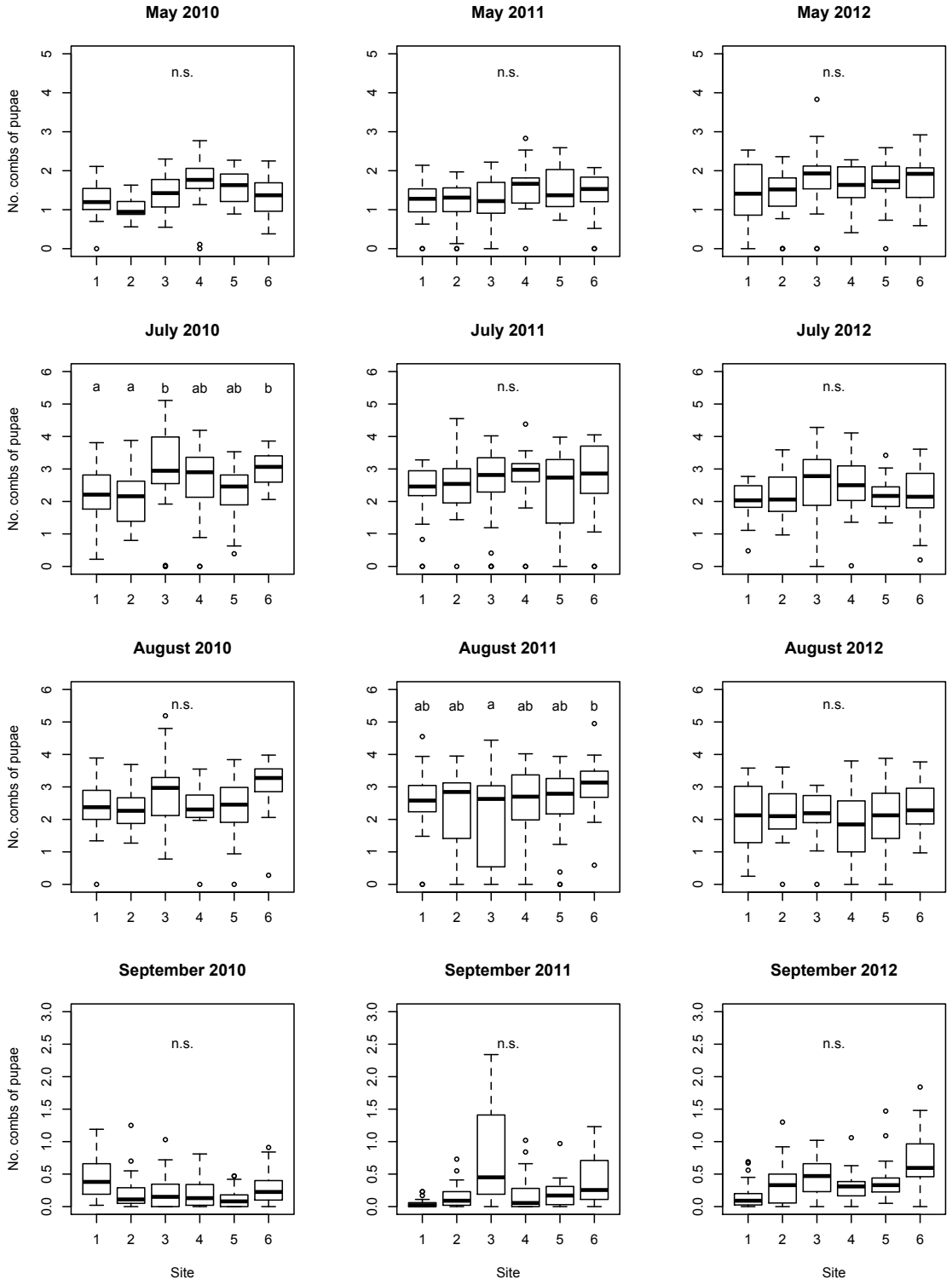

November 2010
November 2011
November 2012
January 2011
January 2012
January 2013
March 2011
March 2012
March 2013
n.s.
No. combs of bees
Site

Figure A2.2


May 2010
May 2011
May 2012
July 2010
July 2011
July 2012
August 2010
August 2011
August 2012
September 2010
September 2011
September 2012
No. combs of pupae
Site
n.s.
a a b ab ab b
ab ab a ab ab b

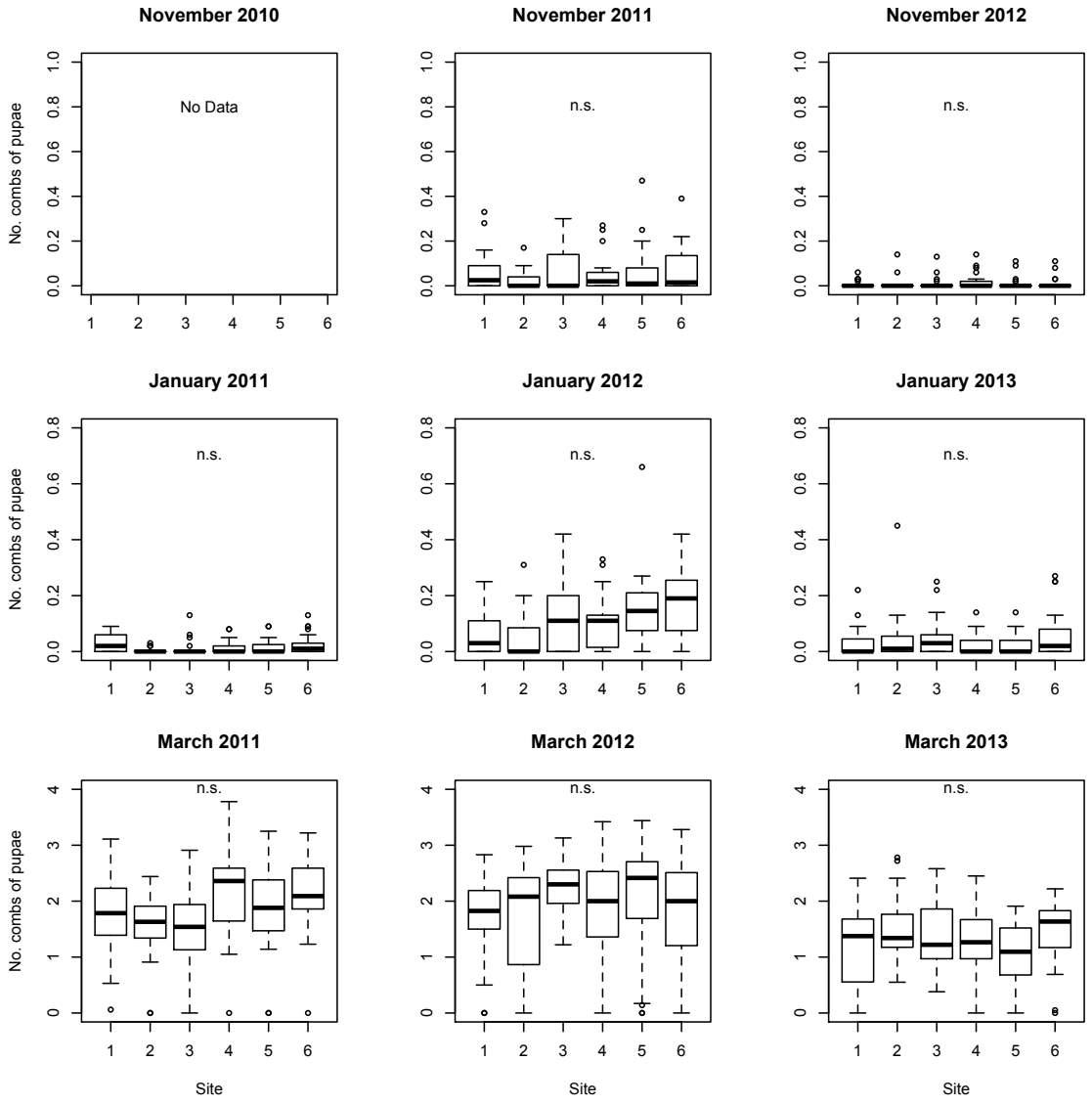

November 2010
No Data
November 2011
n.s.
November 2012
n.s.
January 2011
n.s.
January 2012
n.s.
January 2013
n.s.
March 2011
n.s.
March 2012
n.s.
March 2013
n.s.
No. combs of pupae
Site

Figure A2.3

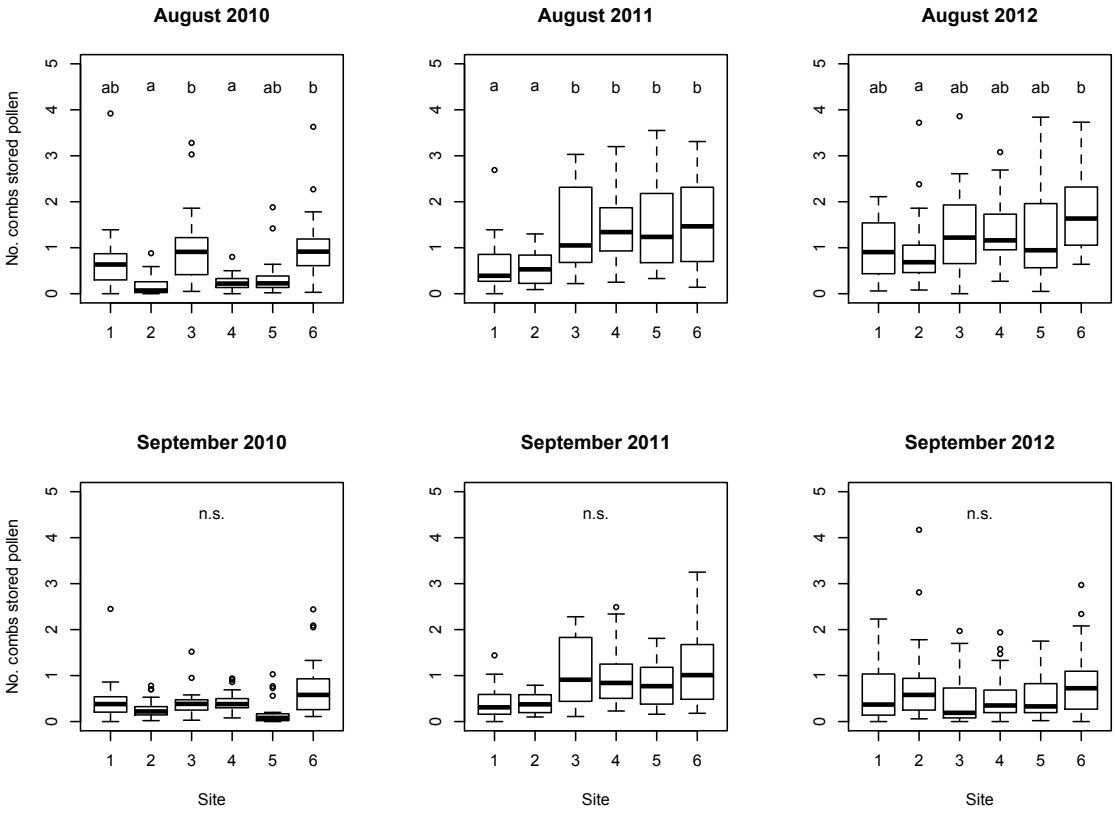
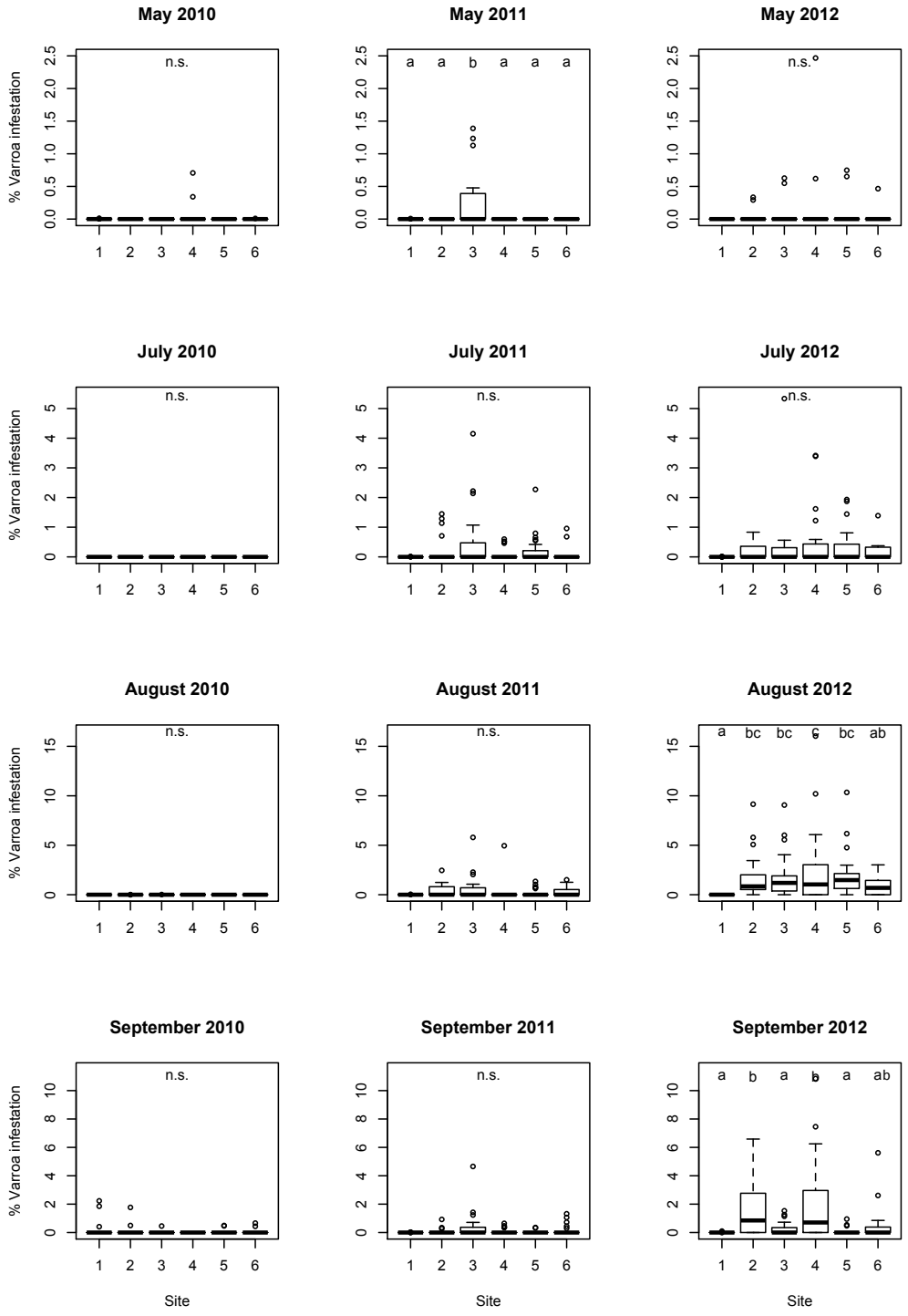

August 2010
August 2011
August 2012
September 2010
September 2011
September 2012
No. combs stored pollen
Site
ab a b a ab b
a a b b b b
ab a ab ab ab b
n.s.

Figure A2.4


May 2010
May 2011
May 2012
July 2010
July 2011
July 2012
August 2010
August 2011
August 2012
September 2010
September 2011
September 2012
% Varroa infestation
Site
n.s.
a a b a a a
a bc bc c bc ab
a b a b a ab

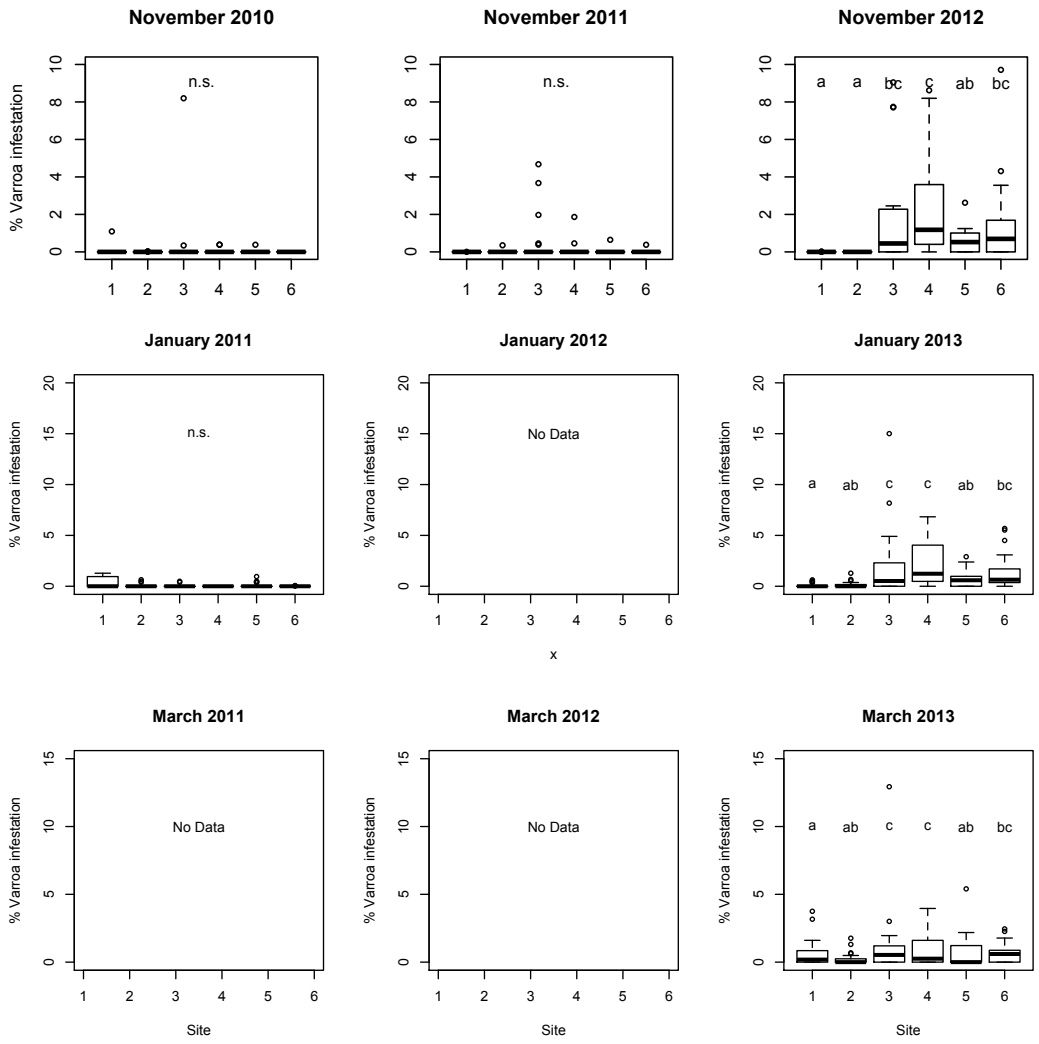
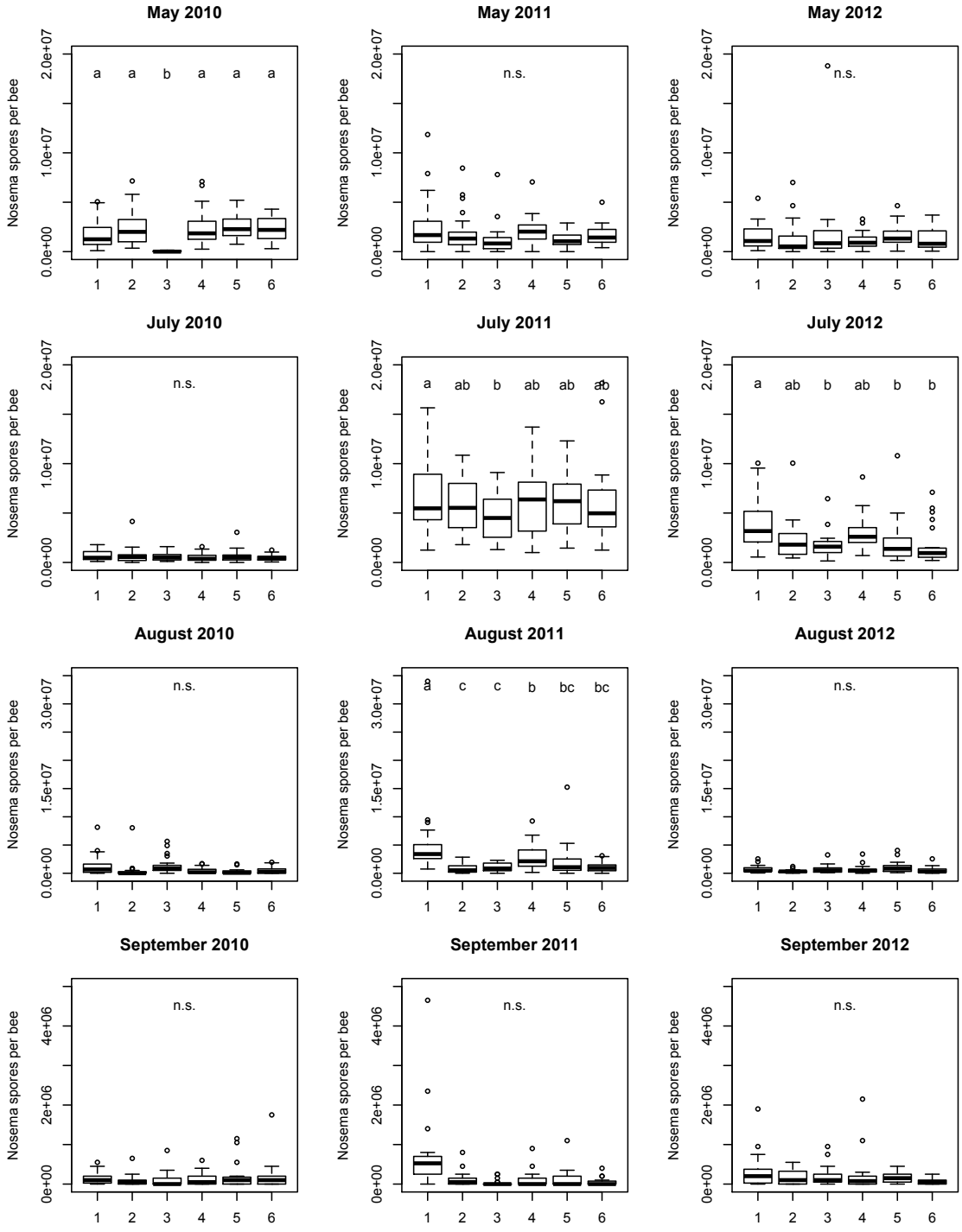

November 2010
November 2011
November 2012
January 2011
January 2012
January 2013
March 2011
March 2012
March 2013
% Varroa infestation
n.s.
No Data
a
ab
bc
c
x
Site

Figure A2.5


May 2010
a a b a a a
May 2011
n.s.
May 2012
n.s.
July 2010
n.s.
July 2011
a ab b ab ab ab
July 2012
a ab b ab b b
August 2010
n.s.
August 2011
a c c b bc bc
August 2012
n.s.
September 2010
n.s.
September 2011
n.s.
September 2012
n.s.
Nosema spores per bee

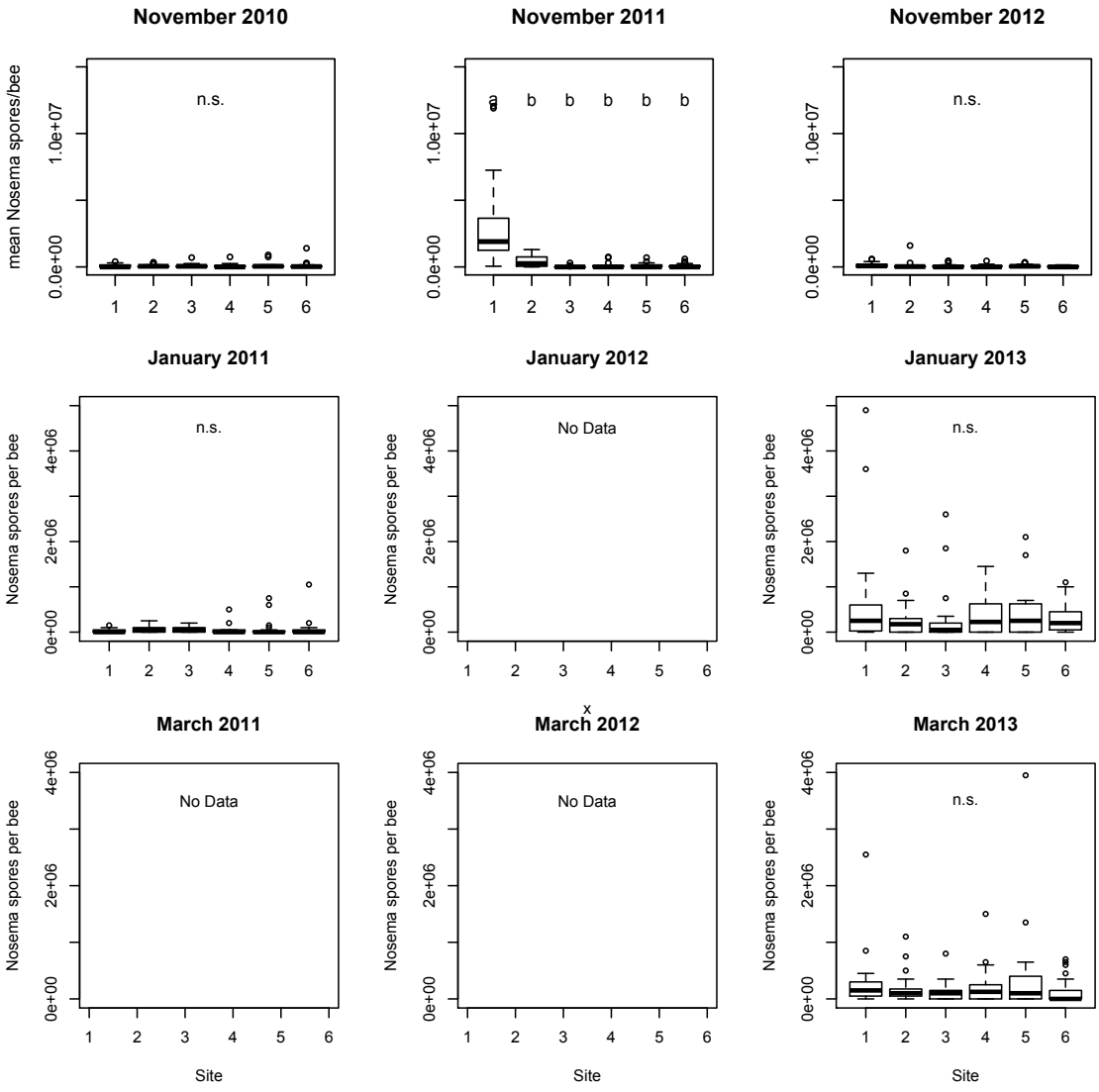

November 2010
November 2011
November 2012
January 2011
January 2012
January 2013
March 2011
March 2012
March 2013
mean Nosema spores/bee
Nosema spores per bee
n.s.
No Data
a b b b b b
0.0e+00
1.0e+07
0e+00
2e+06
4e+06
1 2 3 4 5 6
x
Site

Figure A2.6

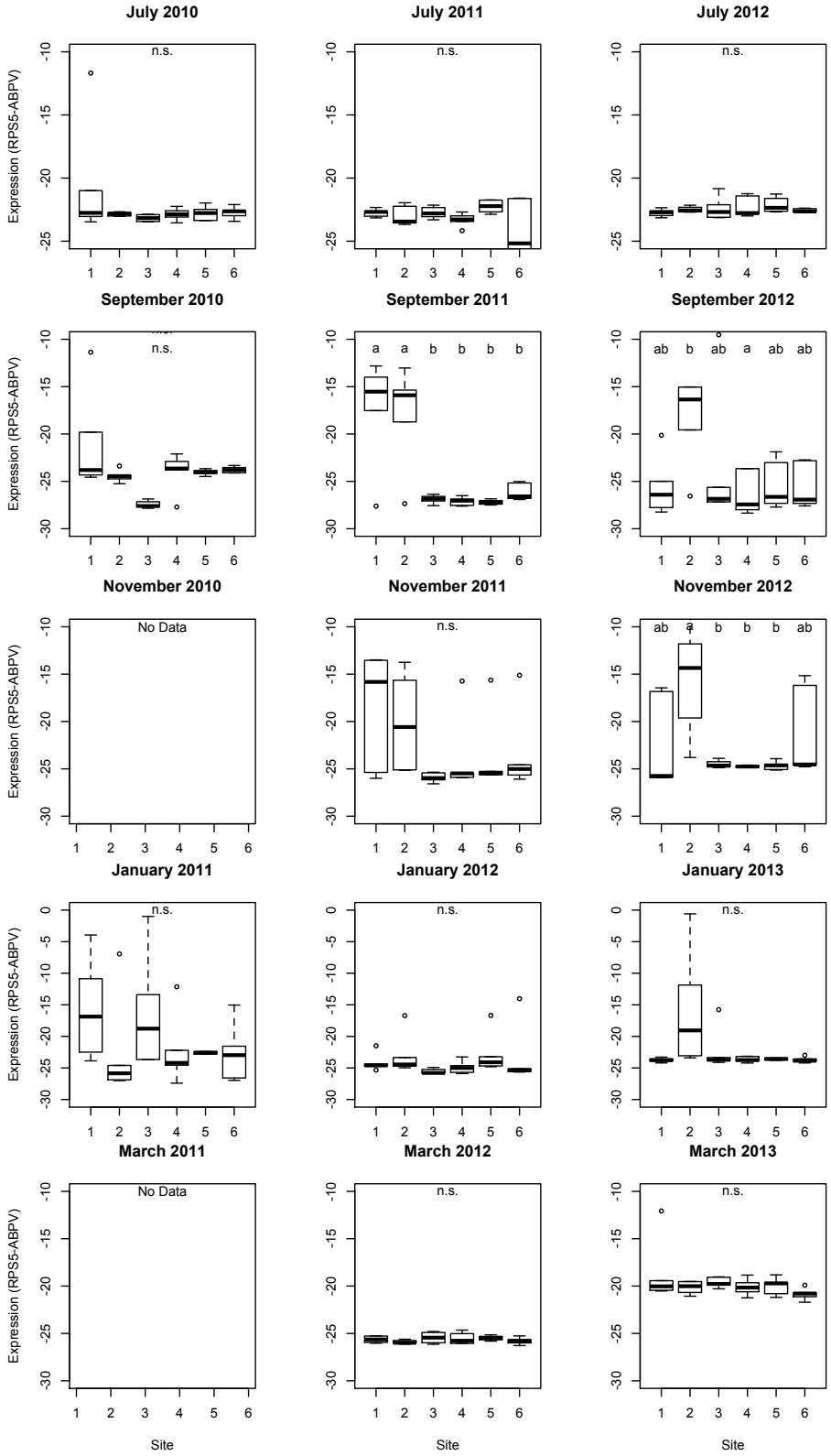

July 2010
July 2011
July 2012
September 2010
September 2011
September 2012
November 2010
November 2011
November 2012
January 2011
January 2012
January 2013
March 2011
March 2012
March 2013
Expression (RPS5-ABPV)
Site
n.s.
No Data
a a b b b b
ab b ab a ab ab
ab a b b b ab

Figure A2.7

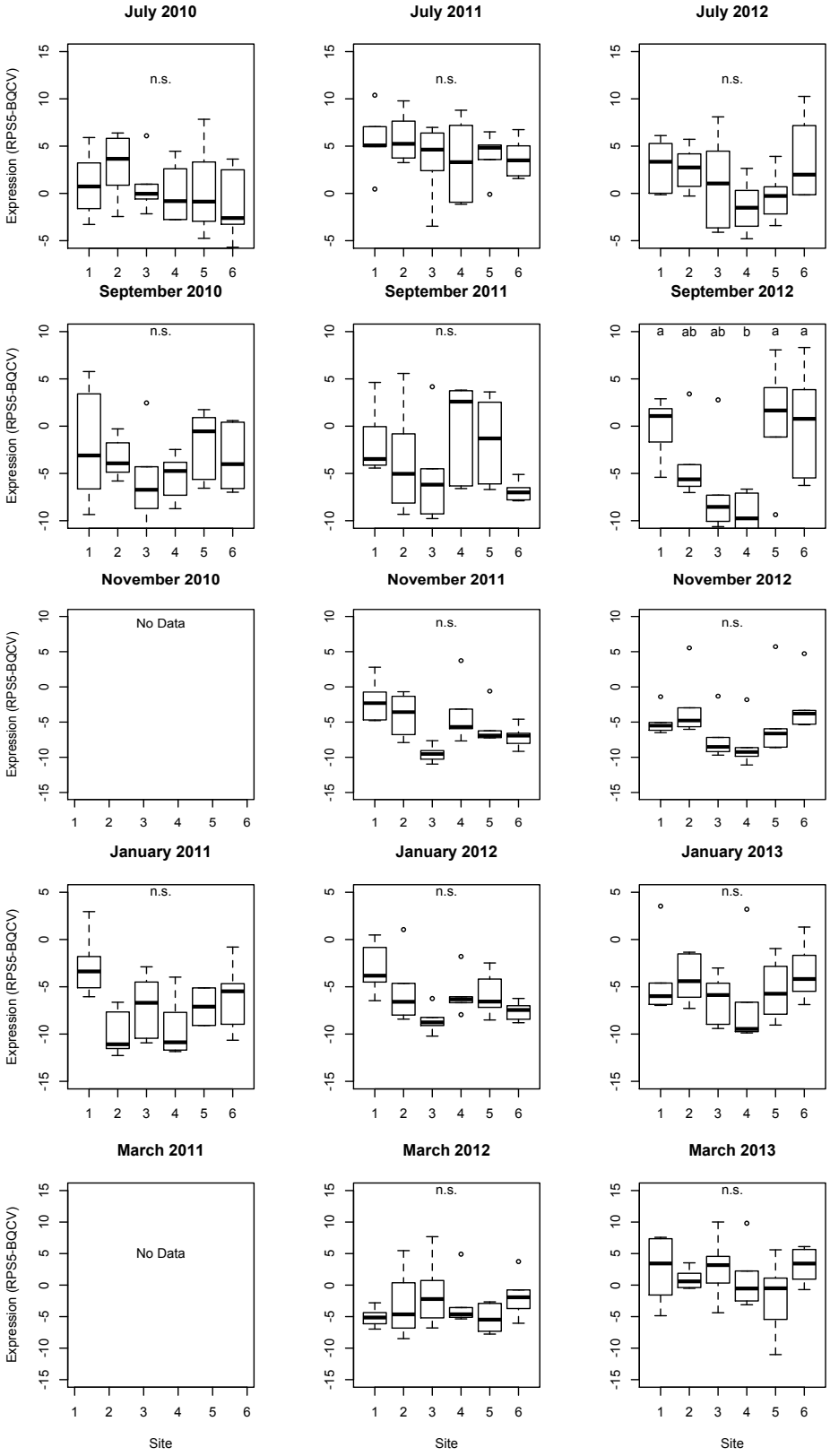

July 2010
July 2011
July 2012
September 2010
September 2011
September 2012
November 2010
November 2011
November 2012
January 2011
January 2012
January 2013
March 2011
March 2012
March 2013
n.s.
No Data
a ab ab b a a
Expression (RPS5-BQCV)
Site

Figure A2.8

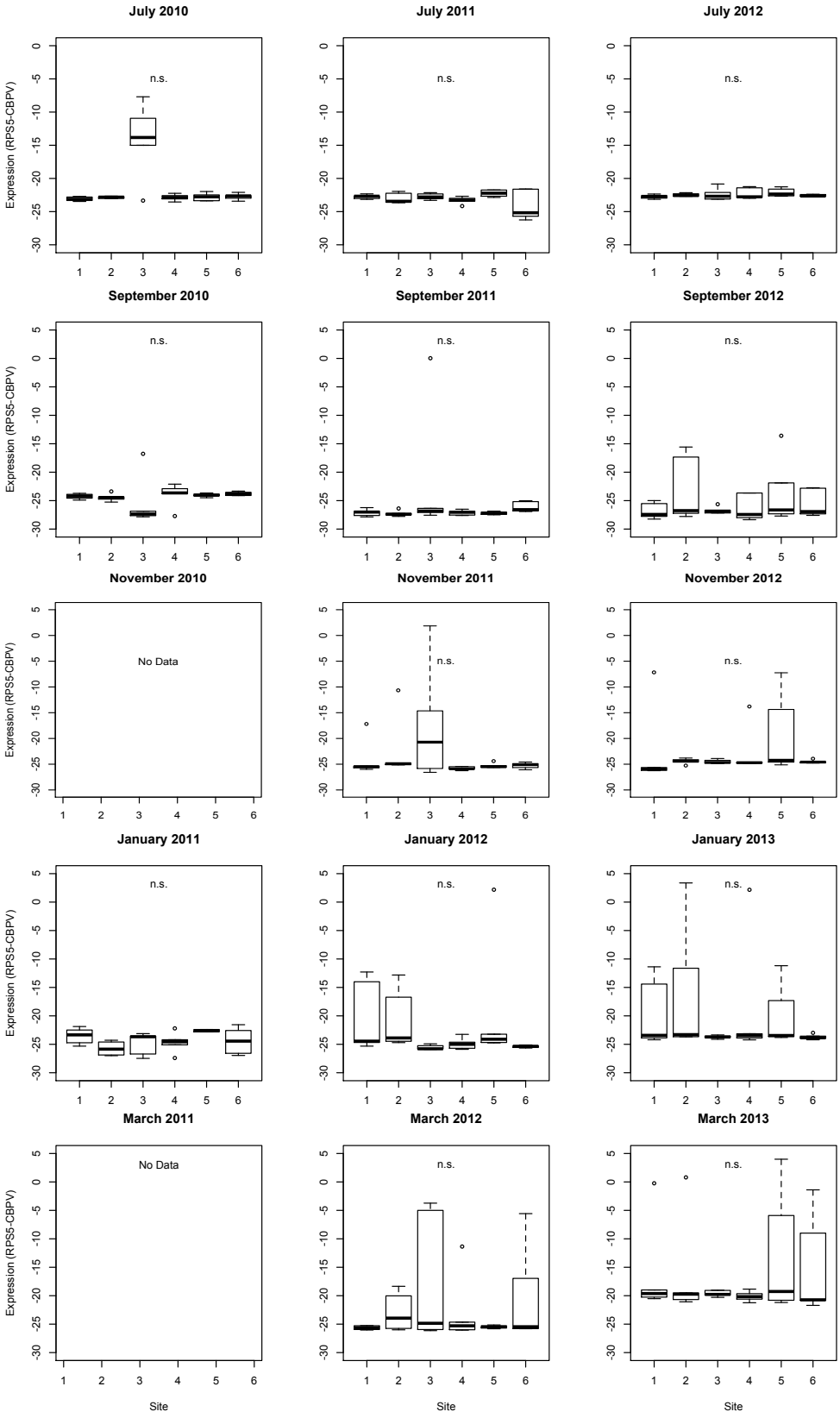

July 2010
July 2011
July 2012
September 2010
September 2011
September 2012
November 2010
November 2011
November 2012
January 2011
January 2012
January 2013
March 2011
March 2012
March 2013
n.s.
No Data
Expression (RPS5-CBPV)
Site

Figure A2.9

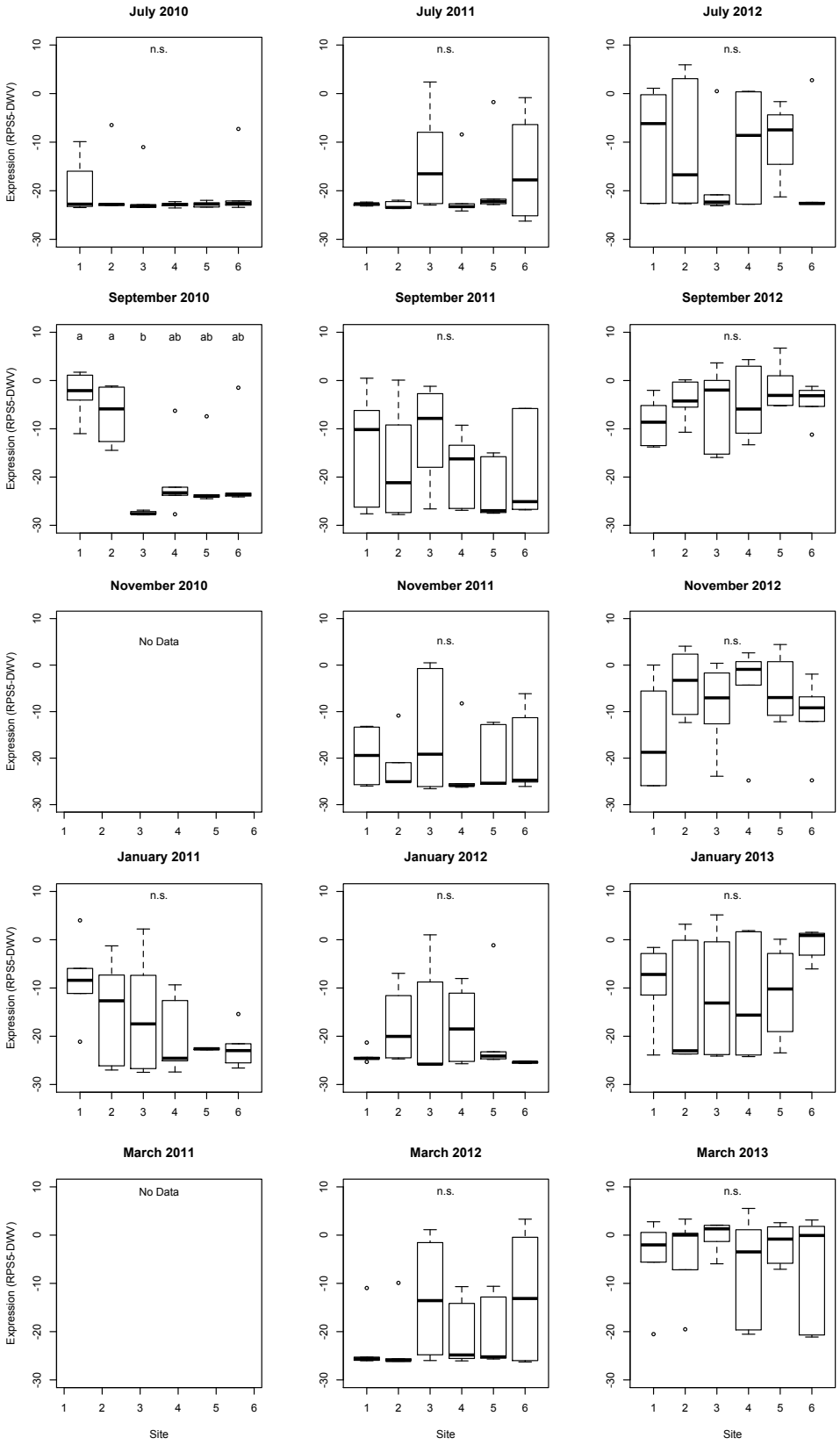

July 2010
n.s.
July 2011
n.s.
July 2012
n.s.
September 2010
a a b ab ab ab
September 2011
n.s.
September 2012
n.s.
November 2010
No Data
November 2011
n.s.
November 2012
n.s.
January 2011
n.s.
January 2012
n.s.
January 2013
n.s.
March 2011
No Data
March 2012
n.s.
March 2013
n.s.
Expression (RPS5-DWV)
Site

Figure A2.10

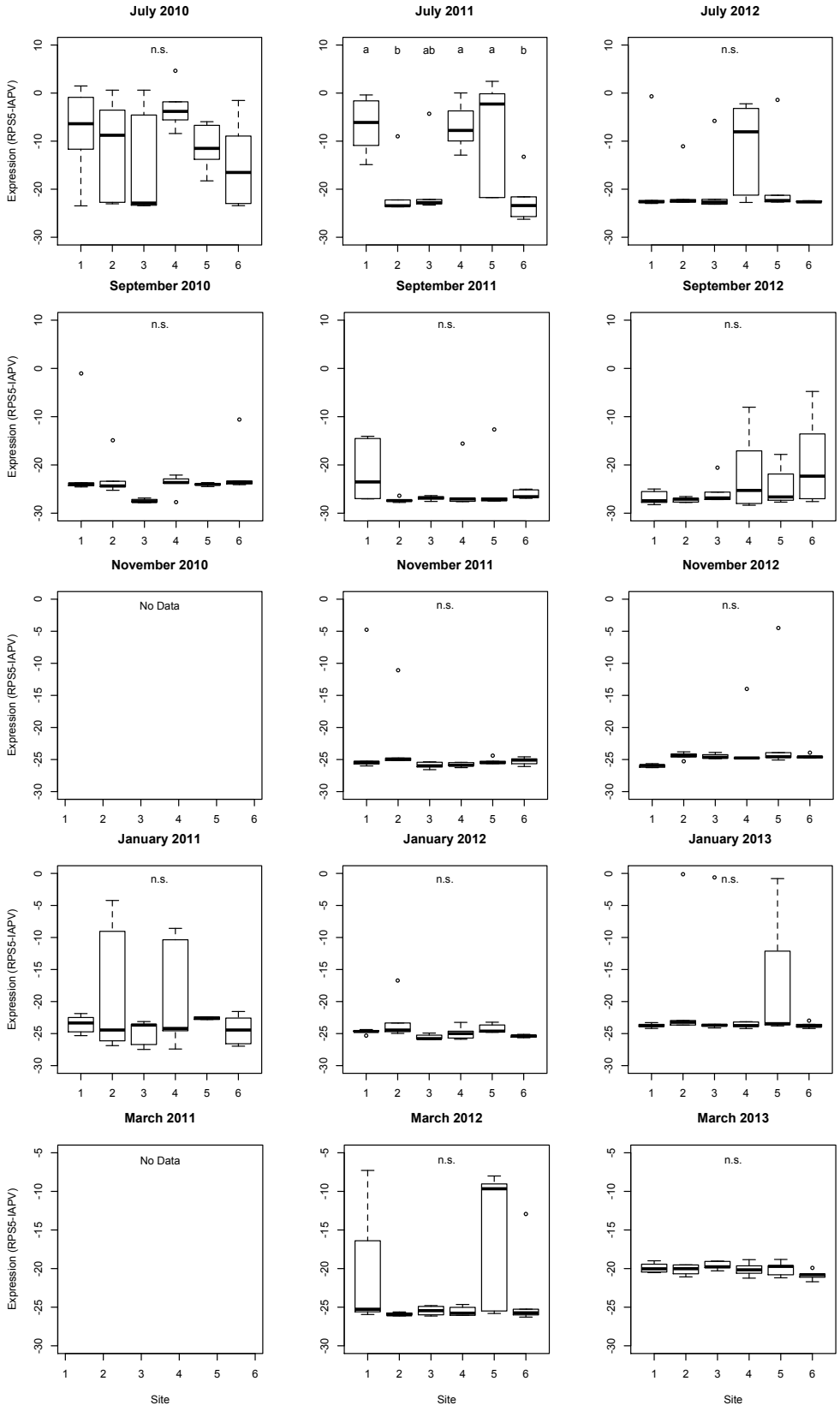

July 2010
n.s.
July 2011
a b ab a a b
July 2012
n.s.
September 2010
n.s.
September 2011
n.s.
September 2012
n.s.
November 2010
n.s.
November 2011
n.s.
November 2012
No Data
January 2011
n.s.
January 2012
n.s.
January 2013
n.s.
March 2011
n.s.
March 2012
n.s.
March 2013
No Data
n.s.
n.s.
Expression (RPS5-IAPV)
Site

Figure A2.11

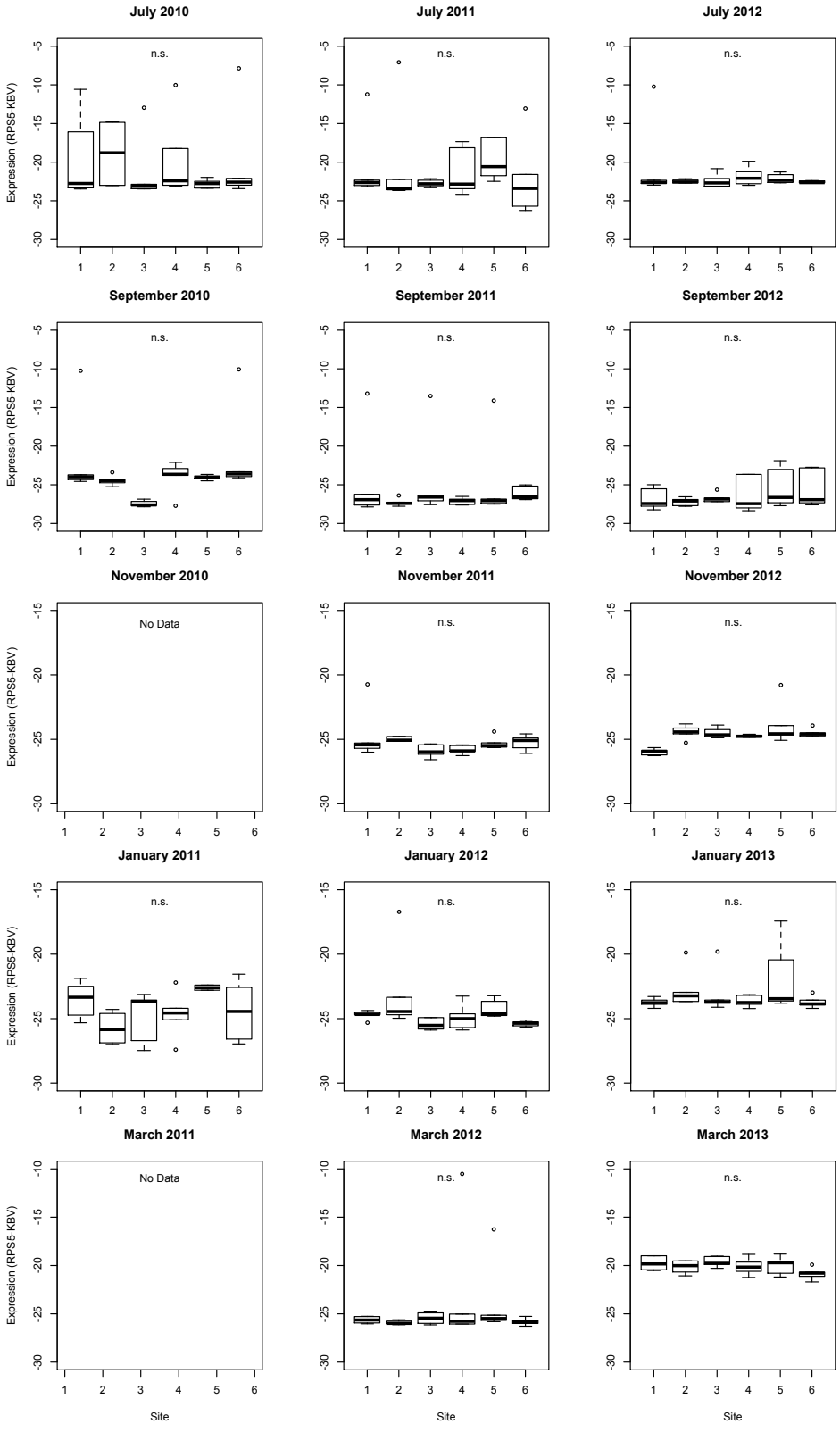
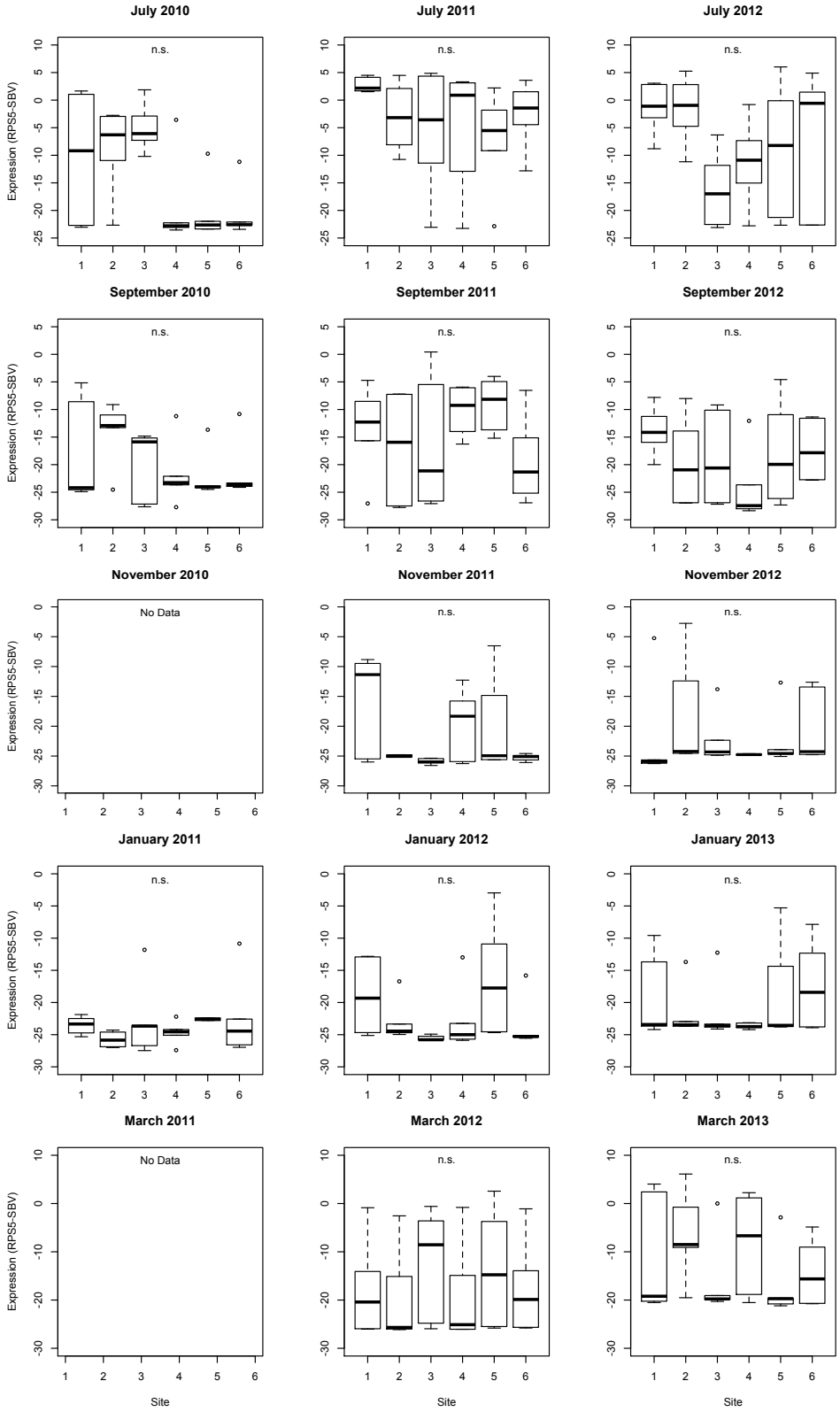

July 2010
July 2011
July 2012
September 2010
September 2011
September 2012
November 2010
November 2011
November 2012
January 2011
January 2012
January 2013
March 2011
March 2012
March 2013
n.s.
No Data
Expression (RPS5-KBV)
Site

Figure A2.12


July 2010
July 2011
July 2012
September 2010
September 2011
September 2012
November 2010
November 2011
November 2012
January 2011
January 2012
January 2013
March 2011
March 2012
March 2013
n.s.
No Data
Expression (RPS5-SBV)
Site